RESISTANT STARCH FROM NOVEL RS-CONTAINING PREPARATIONS: RESIDUAL SUSCEPTIBILITY TO AMYLOLYSIS AND GROWTH OF BIFIDOBACTERIUM STRAINS

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by
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ABSTRACT

Starches differ with respect to both digestion rate and digestion extent in the small intestine. The portion of starch not hydrolyzed by pancreatic α-amylase and absorbed in the small intestine is termed resistant starch (RS). The chemical and physical nature of the starch substrate is a critical factor in whether the starch will be hydrolyzed by pancreatic α-amylase in the small intestine.

Resistant starches differ with respect to both hydrolysis rate and hydrolysis extent in the colon. It is generally assumed that hydrolysis in the colon is due to amylolytic enzymes of the bacterial microbiota. While a number of studies have used in vitro procedures to address the question of which colonic bacterial species are capable of degrading RS-containing materials, there appear to be no in vitro studies designed to address the question of which colonic bacterial species are capable of degrading putative RS obtained from a particular RS-containing material. Although bifidobacteria are believed to play an important role in the breakdown and fermentation of complex carbohydrates that reach the colon, no in vitro studies have been published investigating whether a particular bifidobacterium strain can hydrolyze putative RS in vitro. Due to the lack of experimental data, no information is available on whether the chemical and physical nature of RS may influence RS utilization and fermentability by a particular strain of *Bifidobacterium*. The goal of the present study was to produce and characterize resistant starch-containing preparations with different susceptibilities to pancreatic α-amylase, and compare the growth of selected strains of *Bifidobacterium* when prepared resistant starches with different susceptibility to continued pancreatic α-amylase digestion are used as the main source of carbohydrate.

RS-containing starch preparations were obtained by rehydration (at 1% or 2% starch concentration for 16 hours) in the presence of variable levels of isoamylase (0, 10 or 20U/g of starch) of an ethanol precipitate of either common corn starch (CCS) or high-amylose maize starch (HAMS) that had been molecularly dispersed in 0.5N NaOH. Aspects of physical structure such as crystallinity, helicity, and thermal properties were analyzed using wide-angle X-ray diffraction (WXRD), solid-state $^{13}$C CP/MAS NMR,
and differential scanning calorimetry (DSC). The molecular composition of the RS-containing preparations was analyzed by size exclusion chromatography (SEC) and high performance size exclusion chromatography (HPSEC). The molecular composition of the RS obtained from the RS-containing preparations was also analyzed by HPSEC.

When the ethanol precipitate was rehydrated in the absence of isoamylase, the RS content was not enhanced for the CCS preparations, but this treatment did enhance RS levels somewhat for the HAMS preparations. HPSEC profiles of fully debranched starch obtained by this rehydration treatment of the ethanol precipitates of CCS and HAMS were similar to the profiles of the corresponding CCS and HAMS starting materials, indicating proportional coprecipitation of amylose and amylopectin from dispersion. The lack of crystallinity in the WXRD spectra, the sharpness of the C4 peak (~82 ppm) in the NMR spectra, and the broadness of the endotherm at <140°C in the DSC thermograms suggested the presence of amorphous amylose-amylose double helices and mixed double helices between amylose and amylopectin. Physical structures alone could not explain the difference in enzyme susceptibility. Analysis of the molecular composition of the RS obtained from the RS-containing preparations suggested that the longer double helices in the HAMS samples were more resistant to enzymatic digestion than the shorter double helices in the CCS samples, thus accounting for the difference in RS content between the samples.

Debranching of starch molecules with isoamylase during the rehydration treatment was effective in enhancing RS levels even for CCS. Chemical characterization of CCS and HAMS samples obtained by debranching of the ethanol precipitate during rehydration indicated preferential co-precipitation of amylose and the longer linear chains generated from amylpectin debranching. A greater percentage of crystallinity as observed by WXRD, and the greater enthalpy of melting as observed by DSC for the debranched samples indicated that greater double helical order resulted. Although little increase in crystallinity resulted from HAMS so treated, for CCS the RS content increased with increasing of crystallinity.

For CCS, the sample rehydrated at 2% with 20U isoamylase (2%R-20U) gave the highest starch recovery (48%) and the highest proportion of RS (18%). Therefore, to compare the nature of the preparations obtained, the CCS and HAMS samples rehydrated
at 2% starch concentration with 20U isoamylase were used in further studies. For these two samples, the effect of particle size and thermal treatment on RS content was investigated. The thermal treatment consisted in subjecting the samples to a 30 min boiling treatment in buffer prior to adding \(\alpha\)-amylase for RS determination. The proportion of RS decreased from 35 to 19\% for CCS 2\%R-20U and from 42 to 23\% for HAMS 2\%R-20U, as particle size decreased (from 355<x<550 to x<125 \(\mu\)m). Particle size did not influence the molecular composition of the RS recovered. The proportion of RS after thermal treatment was independent of particle size, about 14\% for the CCS sample and 23\% for the HAMS sample. Molecular composition of the RS indicated a greater proportion of longer double helices in the thermally treated RS than in RS. This outcome was attributed to melting of the shorter chains during the thermal treatment and to double helical association of the then mobile longer chains during the thermal treatment or during cooling.

“Putative” RS was isolated after digestion according to the official method for \textit{in vitro} RS determination (AOAC 2002.02, AACC 32-40). It is considered putative RS because it was obtained by an \textit{in vitro} method. The time course of digestion of RS-containing starches by pancreatic \(\alpha\)-amylase and the residual susceptibility of the putative RS to further pancreatic \(\alpha\)-amylase digestion was determined for the selected novel RS-containing preparations (CCS 2\%R-20U and HAMS 2\%R-20U) and for two commercial RS-containing starches (HAMS and heat-moisture-treated HAMS (HMT)). The time course of digestion data were fitted by using a double exponential decay equation (with 5 parameters) or a triple exponential decay equation (with 6 parameters). Because a limit for pancreatic \(\alpha\)-amylase digestion was not reached within the stipulated 16 h digestion of the official method for \textit{in vitro} determination of RS, the putative RS is susceptible to further pancreatic \(\alpha\)-amylase digestion. The putative RS materials differed in the extent of residual susceptibility. Kinetic analysis of the digestion time course showed that the RS from HMT had the lowest residual susceptibility to pancreatic \(\alpha\)-amylase digestion among the four samples tested.

The presence of residual amylolytic activity associated with the recovered putative RS was observed. The presence of residual pancreatic \(\alpha\)-amylase was observed for all four RS investigated. To decrease a possibly artefactual level of associated
pancreatic $\alpha$-amylase, in subsequent work putative RS was recovered without ethanol precipitation. Whether the residual pancreatic $\alpha$-amylase could make an important contribution to further digestion of RS was investigated by treating the recovered RS as the starch in the official method for *in vitro* RS determination, except that no additional pancreatic $\alpha$-amylase was added to the maleate buffer. Each of the four RS tested was to some extent further digested by the residual pancreatic $\alpha$-amylase.

RS from HAMS was used to determine whether further digestion of putative RS by the action of two levels of residual pancreatic $\alpha$-amylase differently influenced growth and survival for three strains of *Bifidobacterium* (*B. pseudolongum* ATCC 25526, *B. animalis* subsp. *lactis* DSMZ 10140, and *B. animalis* subsp. *lactis* BB12) differing in their ability to degrade starch according to a screening procedure. Further RS digestion due to associated pancreatic $\alpha$-amylase differentially benefited the three strains of *Bifidobacterium* tested.

Because a primary objective of the present thesis was to explore whether bifidobacterium strains could degrade RS, every effort was made to reduce the amylolytic activity associated with the recovered RS without losing integrity of the RS structure. The most effective treatment in reducing the residual amylolytic activity was 24 h incubation at 37°C of the recovered RS (without ethanol precipitation) in 0.1% EDTA solution. Although this EDTA treatment reduced the residual amylolytic activity of the RS recovered in the supernatant by more than 95%, the total level of residual pancreatic $\alpha$-amylase associated with the EDTA treated RS could not be quantified.

Finally, the putative RS from HAMS, HMT, CCS 2%R-20U and HAMS 2%R-20U was recovered, exposed to the EDTA treatment, and used as the main carbohydrate source in MRS medium to evaluate the growth of three strains of *Bifidobacterium* (*B. choerinum* ATCC 27686, *B. infantis* ATCC 15697, and *B. pseudolongum* ATCC 25526) each of which had been shown to partially degrade RS-containing starch according to prior screening. For all three strains, the putative RS from HMT, having the lowest residual susceptibility to pancreatic $\alpha$-amylase digestion, had the least favorable effect on viable cell counts. This outcome indicates that the susceptibility of RS to further digestion may influence the ability of specific *Bifidobacterium* strains to utilize the RS for growth in *vitro*. 
This research shows for the first time that *Bifidobacterium* strains can differently utilize different forms of putative RS *in vitro*. Although it would be inappropriate to conclude about differences in the extent of colonic degradation of the four starches *in vivo*, as other amylolytic activities in the colon are likely to act on each RS, this study represents an important advance in the understanding of RS as substrate for *Bifidobacterium* strains.
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<tr>
<td>1%R</td>
<td>1% starch concentration at rehydration</td>
</tr>
<tr>
<td>10U</td>
<td>10 Unit of isoamylase / g of starch</td>
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<tr>
<td>2%R</td>
<td>2% starch concentration at rehydration</td>
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<tr>
<td>20U</td>
<td>20 Unit of isoamylase / g of starch</td>
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<td>amylglucosidase</td>
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<tr>
<td>EDTA</td>
<td>ethylene diaminetetraacetic acid</td>
</tr>
<tr>
<td>Eq.</td>
<td>equation</td>
</tr>
<tr>
<td>ERS</td>
<td>enzyme resistant starch isolated as described in the official method for <em>in vitro</em> RS determination (AOAC Method 2002.0)</td>
</tr>
<tr>
<td>ERS₁-AMY</td>
<td>enzyme resistant starch with low residual pancreatic α-amylase</td>
</tr>
<tr>
<td>ERS₉L-AMY</td>
<td>enzyme resistant starch with very low residual pancreatic α-amylase</td>
</tr>
<tr>
<td>GIT</td>
<td>gastrointestinal tract</td>
</tr>
<tr>
<td>H1</td>
<td>initial heating</td>
</tr>
<tr>
<td>H2</td>
<td>reheating</td>
</tr>
<tr>
<td>HAMS</td>
<td>high-amylose maize starch</td>
</tr>
<tr>
<td>HMT</td>
<td>heat-moisture treated HAMS</td>
</tr>
<tr>
<td>HPSEC</td>
<td>high performance size exclusion chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>I₂</td>
<td>iodine</td>
</tr>
<tr>
<td>IA</td>
<td>isoamylase</td>
</tr>
<tr>
<td>KI</td>
<td>potassium iodide</td>
</tr>
<tr>
<td>MRS</td>
<td>deMan Rogosa and Sharpe (medium)</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NaN₃</td>
<td>sodium azide</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>No R</td>
<td>non-rehydrated samples</td>
</tr>
<tr>
<td>No-CHO</td>
<td>no carbohydrate</td>
</tr>
<tr>
<td>RS</td>
<td>resistant starch</td>
</tr>
<tr>
<td>SBS</td>
<td>starch binding site</td>
</tr>
<tr>
<td>SCFA</td>
<td>short chain fatty acids</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEC</td>
<td>size exclusion chromatography</td>
</tr>
<tr>
<td>SFS</td>
<td>starch-free supernatant</td>
</tr>
<tr>
<td>SₙD</td>
<td>percentage of starch not digested by pancreatic α-amylase</td>
</tr>
<tr>
<td>SS</td>
<td>sum of squares of the residuals</td>
</tr>
<tr>
<td>TT-ERS</td>
<td>ERS after thermal treatment</td>
</tr>
<tr>
<td>WAXD</td>
<td>wide-angle X-ray diffraction</td>
</tr>
<tr>
<td>WCS</td>
<td>waxy corn starch</td>
</tr>
<tr>
<td>Wt</td>
<td>weight</td>
</tr>
<tr>
<td>λₘₚₓ</td>
<td>maximum wavelength of iodine binding</td>
</tr>
</tbody>
</table>
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Chapter 1 : Literature Review

1.1 Starch

Starch is the major source of carbohydrates in the human diet. Starch makes up to over 50% of the daily energy intake in agrarian cultures and some 25% of westernized societies (Banghurst et al. 1996).

Starch can be obtained from fruits, vegetables, roots and seeds. The main purified food starches used in the United States are corn, tapioca, potato and wheat. Corn starch is the least expensive food starch available in the U.S. and is used in food products both in its native powdered form and in modified forms (Moore et al. 1984).

1.1.1 Chemical structure of starch molecules

Starch is a polymer of glucose, and is made up of two main fractions. The amylose fraction is a mostly linear homopolymer of (1→4)-linked α-D-glucopyranosyl units, having occasional (1→6)-linkages. The precise structure of amylose varies among different plant sources (Hizukuri et al. 1981) and even within a plant source (Takeda et al. 1984). The amylopectin fraction is highly branched, and consists of (1→4)-linked glucose segments connected at their reducing ends by a (1→6)-linkage. Some material has an intermediate structure between the amylose and the amylopectin fractions. This material, referred to as intermediate material, has a molecular weight and amount of branching that is less than amylopectin but greater than amylose (Klucinec and Thompson 1998, Shannon and Garwood 1984).

Several models for the fine structure of amylopectin have been hypothesized (Thompson 2000a). A general view that has emerged since 1972, based on the model first proposed by French (1972), and later modified by Hizukuri (1986), is that the branching of the amylopectin chains is distributed in periodic cluster units. A cluster model of amylopectin is depicted in Fig. 1.1. In this figure, the different types of sub-chains within the amylopectin molecule, termed A, B and C, are also identified.
Figure 1.1 A cluster model of amylopectin (Hizukuri, 1986).
All the lines indicate constituent chains of amylopectin. A chains have a single branching point. B chains have more than one branching point, and are classified into B1, B2, and B3 depending on the number of branching points on the chain.
The A chains tend to be the shortest and are linked to the amylopectin molecules by a single $\alpha$ 1-6 linkage. B chains are identified as those that support A chains and other B chains. B chains are then further classified into B1, B2, B3, and B4 depending on their respective length and the number of clusters they span. There is only one C chain per amylopectin molecule and it is identified as having the only non-reducing end (Hizukuri 1986).

Common corn starch (CCS) contains about 20-25% amylose. The average molecular weight of amylose from CCS is about 250,000 Da (Zobel 1988). The average degree of polymerization (dp) of the component linear chains of amylose is about 950 (Takeda et al. 1988). Takeda et al. (1988) estimated that amylose molecules from CCS have three chains on average.

Amylopectin is the major component of CCS. The amylopectin chains of CCS range from about 18 to 136 dp, with the average chain length of debranched amylopectin about 21 dp (Takeda et al. 1988). The molecular weights of CCS amylopectin is on the order of $10^9$ Da (Zobel 1988). Differences in the MW of amylose and amylopectin fractions are found, resulting from differences in the plant source, the method of polymer isolation, and the method of MW determination (Thomas and Atwell 1999).

Maize starches with > 40% amylose are considered high-amylose maize starches (HAMS) (Shannon and Garwood 1984). Amylose from HAMS has smaller molecular size (average dp between 690-740) and shorter constituent chains (average chain length between 215 and 255 dp) than the CCS amylose (average dp and average chain length between 930-990 and 295-335 dp, respectively) (Klucinec and Thompson 1998, Takeda et al. 1989). Similarly to the amylose from CCS, the amylose from HAMS has three chains on average (Takeda et al.1989).

The amylopectin of HAMS has been shown to be different from the amylopectin in CCS. The amylopectin of HAMS has been reported to have a lower degree of branching, higher average chain length, and a larger proportion of long chains than of CCS (Klucinec and Thompson 1998, Takeda et al. 1993, Takeda et al. 1992a, b).
1.1.1 Physical structures of starch molecules

1.1.1.1 Starch helices

The physical conformation of amylose in dispersion depends on the solvent employed. Banks and Greenwood (1975b) argued that in good solvents such as DMSO, formamide and aqueous alkali, amylose is in the form of a random coil; while, in neutral or alkaline dispersions in the presence of a complexing agent, amylose is largely in a helical conformation. Complexing agents that induce the transition from coil to helix include iodine, fatty acids, alcohols, emulsifiers and a variety of flavor compounds (Buleon et al. 1998, Heinemann et al. 2003). V-amylose is the generic term for amylose in the helical conformation in the presence of the complexing agent. Although the complexing agent is required for formation of the helix, it may not be entirely included in the amylose helices (Buleon et al. 1998).

In V-amylose, the chain conformation is a left-handed single helix. The number of residues per turn varies with the type of complexing agent. In the case of aliphatic alcohols, such as ethanol, the single helix has six residues per turn (V_6) and a pitch of 0.7 nm (Buleon et al. 1998). The outer surface of the single helix is thought to be mostly hydrophilic while the center channel is hydrophobic (Buleon et al. 1998). The interactions that stabilize the helix are intrachain hydrogen bonds between 2-O···O-3 hydrogen bonds between adjacent glucose units, 2-O···O-6 hydrogen bonds between spatially close glucose residues of the next spiral turn (intrahelical hydrogen bonds), and numerous intra- and intermolecular van der Waals contacts (Biliaderis 1998, Immel and Lichtenthaler 2000).

Limited information is available on the formation of single helices from amylopectin. Evans (2005) reported a small proportion of single helices in ethanol-precipitated amylopectin from sodium hydroxide dispersion. Heinemann et al. (2003) reported the formation of single helical inclusion complexes of amylopectin in the presence of lactone as complexing agent.

The external chains of amylopectin and the linear regions of amylose molecules are known to be able to form double helices within starch granules and in non-granular starch (Sarko and Wu 1978). The starch double helix has six residues per turn and a pitch
of 2.13 nm (Buleon et al. 1998, Imberty et al. 1988). Evidence for both right-handed and left-handed double helices has been provided. The left-handed form is the energetically favored form, and it is commonly accepted as the form of starch double helices (Buleon et al. 1998, Imberty et al. 1988). The interactions that stabilize the double helix are hydrogen bonds between glucose units on different strands and numerous van der Waals interactions. The inner portion of the double helix does not accommodate any water molecules (Biliaderis 1998). The outer surface of the double helix has an irregular distribution of hydrophobic and hydrophilic areas (Immel and Lichtenthaler 2000).

Solid-state NMR has been used to probe the short range order of starch chains, in particular certain helical conformations. The different carbons of the glucose monomers have been associated with specific positions in the NMR spectra. Gidley and Bociek (1985) assigned the carbons in the following way: C1 = 98-103 ppm, C2, C3, C5 = 68-73 ppm, C4 = 82 ppm, C6 = 63 ppm. Gidley and Bociek (1985) utilized solid-state NMR to estimated the proportion of double helices in granular starches by comparison of the NMR spectra of the starches with model spectra obtained by superposition of the NMR spectra of double helical and amorphous reference samples. Bogracheva et al. (2001) and Atichokudomchai et al. (2004) suggested that the partial peak area (PPA) of the peak at ~82 ppm could be used to estimate the proportion of double helices in the samples as:

\[ 100 - \frac{\text{PPA at 82 ppm of sample}}{\text{PPA at 82 ppm of amorphous reference}} \]

1.1.2.2 Crystalline structures

Association of helices formed by starch molecules can form crystalline arrays. The arrangement of the packing of the helices determines the crystallinity pattern as observed by wide-angle X-ray diffraction (WAXD).

In the characteristic structure of crystalline V-amylose, helices are packed with either orthorhombic or hexagonal symmetry (Biliaderis 1998). The crystalline V form exists in anhydrous (V_a) and hydrated (V_h) states (Fig. 1.2). Even though the surface of the inner cavity of the single helix is hydrophobic, intrahelical water has been found in the anhydrous V_a form as well as in the hydrated V_h form. The transition of anhydrous to
hydrated form occurs at $a_w$ 0.6, and the number of water molecules increases from 4 to 16 per unit cell (Guilbot and Mercier 1985).

Double helical starch can form two different crystalline polymorphs. The A-pattern consists of starch double helices packed into a monoclinic array (Fig. 1.3). Eight water molecules are present per unit cell (Imberty et al. 1988). The B-pattern is a more open and hydrated structure, consisting of double helices packed in a hexagonal array. Thirty-six water molecules per unit cell fill the large central channel formed by the hexagonically packed double helices (Fig. 1.3); about half of the water molecules are tightly bound to the chains, and the other half is connected only to other water molecules (Imberty and Perez 1988).

Analyses by WAXD (Le Bail et al. 1995, Zaslow and Miller 1961) and by high resolution solid-state $^{13}$C NMR (Saito et al. 1991) showed that hydration of V-amylose results in the at least partial conversion to double helices in B-type polymorphs. The conversion to pure B-type was observed for V-amylose prepared from amylose with DP 1000 or DP 17, while the conversion to mixture of V- and B-type polymorphs was observed for V-amylose prepared from amylose with DP 100 (Saito et al. 1991). The transition from the single-helical V-type to the double helical B-type has been explained as a transient disappearance of the V-type crystallinity and subsequent reorganization to double helices when moisture was added to the samples (Le Bail et al. 1995, Zaslow and Miller 1961). The V-type starch composed of starch-lipid complexes has been found to be stable upon rehydration (Kawada and Marchessault 2004).
Figure 1.2 X-ray diffraction patterns of V_a and V_h-amyloses along with the (a,b) plane projections of helices, showing the hydrogen bonding arrangement (Biliaderis 1998).
Figure 1.3 Projection of the crystalline structures onto the \((a,b)\) plane for the A-type crystallinity (top) and B-type crystallinity (bottom), showing the packing arrangements of double helices, water molecules (black spots), and hydrogen bonding (dashed lines). Models taken from Imberty et al. (Imberty et al. 1988, Imberty and Perez 1988).
1.1.2.3 Granular structure

The morphological features of starch granules vary with the plant source. CCS granules have a polyhedral and rounded shape, with average diameter about 15 μm. HAMS granules are generally smaller (average diameter about 5 μm) and have a less regular shape than CCS granules (Thomas and Atwell 1999, Zobel 1988).

Molecular order in native starch is evident from the birefringence pattern (Maltese cross) observed in granules when viewed under cross-polarized light. The alternated bright and dark quadrants indicate radially aligned molecules. Birefringence is commonly thought to be due to radial orientation of the external chains of amylopectin in the granule. However, the strength of the birefringence is not necessarily proportional to amylopectin content (Evans et al. 2003).

Starch granules are partially crystalline, with crystallinity generally in the range of 15 to 45% (Parker and Ring 2001, Zobel 1988). The crystallinity of the granule is thought to be associated with the external chains of amylopectin. The external chain lengths determine the degree of crystallinity (Hizukuri 1985). Amylopectin crystallites are radially symmetrical in the granules. The chains are arranged into alternating crystalline and amorphous lamellae with a periodicity of 9 to 10 nm (Jenkins et al. 1993). In the crystalline lamellae, the chains are associated in double helices and are packed together in an array to form clusters. In the amorphous lamellae, the branch points reside. The starch granule is also known to contain relatively amorphous regions alternating with relatively crystalline regions (Gallant et al. 1997). The term “growth rings” has been suggested to describe comprising concentric rings 120-140 nm thick of alternating semi-crystalline and amorphous regions, which can be observed by optical microscopy (French 1984, Jenkins and Donald 1995).

Evidence from various microscopic techniques, as well as from enzymatic degradation studies suggests that the crystalline and amorphous lamellae of the amylopectin molecules may be associated into larger spherical structures termed “blocklets” (Gallant et al. 1997). Gallant et al. (Gallant et al. 1997) proposed that the crystalline regions consist of larger blocklets (50 to 500 nm) than the amorphous regions, where the blocklet size ranges between 20 and 50 nm.
In contrast to the defined location and role of amylopectin, the location and role of amylose within the granule is poorly defined. It has been hypothesized (Jenkins and Donald 1995, Zobel 1988) that the amorphous growth rings may contain predominately amylose and less ordered amylopectin, and that the interaction between amylose and amylopectin in these amorphous regions may be the cause of the decreased crystallinity. A number of studies suggest that the location of amylose with respect to the amorphous and/or crystalline regions depends on the botanical source of the starch (Gerard et al. 2002, Jane and Shen 1993, Oates 1997, Saibene et al. 2008)

1.1.2.4 Structures formed during retrogradation

Starch occurs naturally as water-insoluble granules. Molecular dispersion is achieved when the constituent starch molecules are dispersed; both amylose and amylopectin are brought into dispersion (Banks and Greenwood 1975a). Molecular dispersion of the native starch granules can be achieved by heat and water, or by solvent. In many common thermal treatments, it is likely that some residual molecular structure remains (Fisher and Thompson 1997). Polymer/solvent interactions may be used to disperse the starch and overcoming the original molecular order.

The interaction of the starch molecules and the solvent relative to the interactions of the starch molecules with other starch molecules dictate the stability of the starch molecules in a given solvent. In a good solvent, the starch-solvent interactions are preferred to the starch-starch interactions and the starch molecules tend to have a more extended conformation (Rees et al. 1982). An example of good solvent for starch molecules is sodium hydroxide. When starch is dispersed in a sodium hydroxide solution, the hydroxyl groups, which are mildly acidic pK~ 12 (Foster 1965), are negatively charged, leading to electrostatic repulsion between starch molecules. Therefore, in sodium hydroxide both amylose and amylopectin are dispersed fully.

Water is a poor solvent for starch molecules. In water, the starch-starch interactions are preferred to starch-solvent interactions, and the starch molecules do not disperse in water at room temperature. Starch molecules that have been dispersed are not stable in water at room temperature and tend to aggregate and precipitate.
Amylose precipitation from aqueous solution by molecular association has been intensively studied (reviewed in Foster 1965). Double helices and aggregated double helices are associated with this retrogradation behavior (Gidley and Bulpin 1989, Klucinec and Thompson 1999). Double helices may aggregate and organize into crystallites (Miles et al. 1985, Ring et al. 1987). Two of the most important variables that affect the molecular association of amylose are polymer concentration and chain length (Adkins and Greenwood 1966, Biliaderis 1998). Amylose aggregation in aqueous systems is enhanced by increasing the concentration (Adkins and Greenwood 1966). Amylose molecules with chain length of about 100 are the least stable in aqueous systems (Gidley and Bulpin 1987).

Precipitates of short linear chains exist as double helices (Biliaderis 1998). The minimum chain length required for double helix formation at room temperature is 10 (Gidley and Bulpin 1987). Buleon et al. (1984) showed that for amylose dispersed in water (0.05% concentration) by autoclaving, the crystalline polymorph of the precipitate depends on the water proportion of the dispersion and the temperature of crystallization. B-type polymorphs are obtained by precipitation of amylose from water, while A-type polymorphs are obtained by precipitation of amylose from a mixture of water:ethanol (15:85). They also observed that when the proportion of ethanol in the water:ethanol mixture is increased to roughly 50:50 or higher, the V-type polymorphs are obtained (Buleon et al. 1984).

Amylopectin molecules in dilute aqueous dispersions are far more stable than amylose (Adkins and Greenwood 1966, Zobel 1988). However, amylopectin dispersions with concentrations above ~0.9% (w/v) exhibit slowly progressive aggregation (Adkins and Greenwood 1966, Banks and Greenwood 1975a). Precipitates of amylopectin are mainly in the form of double helices (Biliaderis 1998, Buleon et al. 1998). Both interchain association, between external chains on different molecules, and intrachain association, between external chains in close proximity on the same molecule, are presumably responsible for aggregation of amylopectin in form of double helices (Biliaderis 1998, Buleon et al. 1998). The possibility of chains participating in interchain double helices would be limited to chains that have the potential to be in proximity to other chains from other molecules (Klucinec and Thompson 1999).
Mixtures of amylose and amylopectin in aqueous dispersions at starch concentrations >1.5% have been investigated mainly upon cooling with the purpose of gaining insight to the formation of starch gels (Biliaderis 1998). The contribution of amylose and amylopectin to molecular aggregation from dispersion is still unclear. Phase separation of amylose and amylopectin at 80°C has been documented by Kalichevsky and Ring (1987). Other studies (Biliaderis 1998, Doublier and Llamas 1993, Leloup et al. 1991) supported the incompatibility of the two starch molecules in an aqueous medium, leading to phase separation, with one phase being enriched in amylose and the other containing mainly amylopectin. Some studies of gelled mixtures of amylose and amylopectin suggest the interaction between amylose and amylopectin, leading to mixed amylose-amylopectin physical junction zones (Adkins and Greenwood 1966, Gudmundsson and Eliasson 1990, Klucinec and Thompson 1999, Klucinec and Thompson 2002, Rindlav-Westling et al. 2002). Gudmundsson and Eliasson (1990) and Rindlav-Westling et al. (2002) suggest that amylose and amylopectin co-crystallization occurs to a greater extent when amylose is present in greater proportion than amylopectin. This observation is supported also by Aberle and Burchard (1997), who showed that starch molecule aggregation in semidilute aqueous dispersions (between 5 to 30% (w/v)) is promoted by the addition of amylose. The onset of aggregation was found to be at lower concentrations as the amylose content in dispersion increased.

1.2 Starch hydrolysis

1.2.1 Enzymes

The term “amylase” can be generally defined as a category of enzyme which hydrolyzes the O-glycosyl linkage of starch (Kuriki and Imanaka 1999). Amylases are widely produced by plants, bacteria and animals (Yamamoto et al. 1995). They are classified according to the following criteria: 1) the configuration of the anomeric carbon of their products (α versus β); 2) the action mode on the substrate (endo or exo); 3) the linkage attacked (α(1→4) and/or α(1→6) linkage); 4) the type of product(s) produced (glucose, maltose, dextrins, etc.); 5) the protein structure; and 6) the biological source
The older and still most common classification of amylases is as either \( \alpha \) or \( \beta \) designation, based on the anomeric configuration of the product(s) released.

The \( \alpha \)-amylases are the most widely distributed of the amylases; they are produced by many different types of bacteria, fungi, animals, and some plants. \( \alpha \)-amylases are generally endo-acting enzymes. There are also \( \alpha \)-amylases that hydrolyze starch polysaccharides to products with the \( \alpha \)-anomeric configuration and are exo-acting. The shape of \( \alpha \)-amylases is globular (Desseaux et al. 1988), with an approximate diameter of 6 nm (Payan et al. 1980).

The \( \alpha \)-amylase family is comprised of enzymes with almost 30 different specificities, comprising hydrolases, transglycosidases and isomerases (MacGregor et al. 2001). However, all the members of the \( \alpha \)-amylase family show structural similarity and a common catalytic mechanism (Kuriki and Imanaka 1999). Three domains (domain A, B and C) are found in all the members of the family (Fig. 1.4). The largest is domain A, which consists of a \((\beta/\alpha)_8\)-barrel, i.e., a barrel of eight parallel \( \beta \)-strands surrounded by a concentric cylinder of \( \alpha \)-helical segments (Brayer et al. 1995). Domain A is believed to contain the active site region.

The active site of an enzyme belonging to the \( \alpha \)-amylase family is considered to be made up of a number of subsites, each subsite capable of a binding interaction with one glucose residue of the substrate. The subsites themselves are composed of side chains of amino acid residues situated on loops in the enzyme structure that connect the C-terminal ends of \( \beta \)-strands to the N-terminal ends of the adjacent helices of the \((\beta/\alpha)_8\)-barrel of the catalytic domain. Because the architecture of the \( \beta/\alpha \) loops varies from enzyme to enzyme, the number and nature of the subsites at the active site also varies, and is characteristic of a particular enzyme (MacGregor et al. 2001).
Figure 1.4 Schematic stereooptical representation of human pancreatic \( \alpha \)-amylase (Brayer et al. 1995).

A central feature of this structure is the \((\beta/\alpha)_8\)-barrel that forms the bulk of Domain A and contains the active site region. The location of the calcium and chloride ions and the N- and C-terminal ends of the polypeptide chain have also been labeled N and C, respectively.
1.2.2 Pancreatic α-amylase

Pancreatic α-amylase is an *endo*-type α-amylase, which catalyzes the hydrolysis of internal α-(1,4)-glucosidic bonds in amylose and amylopectin, resulting in a mostly random, multiple attack of the substrate toward the non-reducing end (Colonna et al. 1992, Robyt 1984). In this model, most interior glycosidic linkages in the long linear chains have the same initial probability of being hydrolyzed (Colonna et al. 1992). After hydrolysis, the part of the macromolecular chain containing the reducing terminal is retained by the enzyme and the chain is repositioned usually at least two glucose units along the active site, enabling a new hydrolysis toward the non-reducing end (Mazur and Nakatani 1993).

Porcine pancreatic α-amylase is highly similar in structure and action to human pancreatic α-amylase. Porcine pancreatic α-amylase is a continuous polypeptide chain of 496 amino acids (Kluh 1981), with a molecular weight of about 55,000 Da (Desseaux et al. 1988). The active site of porcine pancreatic α-amylase has been postulated to contain 5 subsites, with the catalytic site located between the second and third subsite from the reducing end subsite (Robyt 1984). In order to be hydrolyzed, the substrate has to fit into the active site cleft, and the glucose units must form energetically favored non-covalent interactions with the amino acid chains of the subsites. Essentially no glucose is produced from amylose or amylopectin by the action of this enzyme. Maltose, maltotriose, and maltotetraose are the main products of amylose hydrolysis. In addition to the products mentioned for amylose digestion, branched α-limit dextrins are formed from hydrolysis of amylopectin (Robyt 1984).

Pancreatic α-amylase cannot hydrolyze the α-1,6 linkages of amylopectin. Even the glucose units close to the branch points have less favorable binding energy with the subsites of the enzyme than the glucose units further down the chain. Variation in subsite binding therefore accounts for the efficiency with which the enzyme hydrolyzes a linkage close to a branch point in amylopectin (Mazur and Nakatani 1993). Internal linear chains of amylopectin can only be hydrolyzed by α-amylase if the chain is sufficiently long to allow favorable binding of the enzyme to the subsites.
The architecture of pancreatic α-amylase consists of the three domains typical of the α-amylase family (domain A, B, and C). A calcium ion is tightly bound between domain A and B (Fig. 1.4) (Larson et al. 1994). The role of the calcium binding site of human α-amylase is to stabilize the structure of domain B (Brayer et al. 1995, Robyt 1984). Tightly bound near the active site (on domain A) the enzyme also contains a chlorine ion, that may serve as an activator for catalysis (Brayer et al. 1995, Larson et al. 1994). The presence of these calcium and chlorine ions is essential for full catalytic activity (Desseaux et al. 1988, Sky-Peck and Thuvasethakul 1977).

Ethylenediaminetetraacetic acid (EDTA) is a chelating agent used to cause the dissociation of the calcium ion from the active enzyme molecule, reversibly inactivating the enzyme (Lecker and Khan 1996, 1998, Sky-Peck and Thuvasethakul 1977). The inactivation of the enzyme becomes irreversible if the EDTA treatment is done at temperature about 40°C (Lecker and Khan 1996, 1998). The mechanism involved in the irreversible inactivation of α-amylase in pure solution by EDTA consists in a reversible inactivation caused by the dissociation of the calcium ion from the active enzyme molecule, followed by a second irreversible inactivation in which the apoenzyme undergoes thermal denaturation (Lecker and Khan 1996, 1998).

Binding studies (Alkazaz et al. 1996, Qian et al. 1995) have shown the existence of a site at the surface of the molecule where the enzyme can bind to the substrate; the so-called starch binding site (SBS). Although an SBS is inferred for pancreatic α-amylase, no information is available about the SBS of pancreatic α-amylase. However, binding studies on α-amylases of microbial origin suggest that SBD is a comprised of a planar aromatic surface, which is appropriate for binding to α-1,4-linked glucoses. This site is proposed to act as the initial recognition site for starch (Sorimachi et al. 1997), by recognizing the surfaces of the relatively rigid helical structures of starch (Abe et al. 2004).

1.2.3 Limiting factors of starch hydrolysis

To understand factors that may influence enzymatic starch hydrolysis, four aspects of hydrolysis should be considered: the diffusion of the enzyme towards the substrate, the porosity of the starch substrate, the adsorption of the enzyme on the
substrate, and the catalytic event (Colonna et al. 1992). For native starch granules the limiting factor for the hydrolysis has been shown to be the penetration of the enzyme into the granules by successive formation of pits and larger pores (Gallant et al. 1973). For non-granular starch materials, several limiting factors for the hydrolysis have been suggested, including: amylose/amylopectin ratio; substrate crystallinity; degree of gelatinization; particle size; extent of retrogradation; presence of other components interacting with starch such as amylose-lipid complexes and starch protein interactions (Colonna et al. 1992).

The physical nature of the starch substrate is a critical factor to determine whether the starch will be hydrolyzed. First, the enzyme must have physical access to the region in which the catalytic action can occur. If the pores in a polymer network are so small that the enzyme is excluded, then the chains in the network are effectively indigestible (Colonna et al. 1992, Gidley et al. 1995). Even when the enzyme has physical access to a starch chain, a sufficient length of the glucose units must be able to physically conform to the binding subsites that make up the active site of the enzyme. If starch flexibility is constrained, a possible hydrolysis event in that region may be slower or precluded. Structures such as double helices are in a wrong conformation to fit into the active site cavity of pancreatic α-amylase, and can only be hydrolyzed in their unwound state (Colonna et al. 1992).

Enzyme hydrolysis of starch has been shown to be fast in the beginning and then slower with increasing digestion time (Brumovsky and Thompson 2001, Evans and Thompson 2008, Planchot et al. 1997, Rees 2008). Englyst et al. (1992) introduced the terms “readily digestible starch” and “slowly digestible starch” to describe the proportion of starch digested at two time points during digestion. Evans and Thompson (2008) performed a kinetic analysis of digestion for granular and non-granular starch samples. They reported the presence of two components of digestion with different rate constants of digestion, and attributed these two components to two separate substrates. They suggested substrate 1 to be composed of only amorphous material, and substrate 2 to contain both amorphous and crystalline structures.
1.3 Starch digestion and absorption in the human

Digestion of starch by humans involves salivary $\alpha$-amylase and pancreatic $\alpha$-amylase. Salivary $\alpha$-amylase is the first amylase that comes in contact with the ingested food. Given the short residence time of food in the mouth, starch is only hydrolyzed to a limited extent by salivary $\alpha$-amylase. The salivary $\alpha$-amylase quickly passes from the mouth to the stomach together with the food (Groff and Gropper 2000).

After some residence time in the stomach, the partially hydrolyzed starch passes into the small intestine. The main hydrolysis of the starch is then accomplished by pancreatic $\alpha$-amylase. Digestion of the smaller branched and linear products generated by the action of pancreatic $\alpha$-amylase is completed at the brush border surface of enterocytes by two disaccharidases. The $\alpha$-1,4-glucosidase (so-called “maltase”) converts maltose, maltotriose, and maltotetraose into D-glucose by successive action from the non-reducing end (Robyt, 1984). The $\alpha$-1,6-glucosidase (so-called “isomaltase”) hydrolyses the $\alpha$-D-(1→6) linkages (Robyt, 1984). The resulting D-glucose is then actively transported across the lumenal membrane of the small intestine and then into the blood (Groff and Gropper 2000). However, some starches have been found to be partially resistant to pancreatic $\alpha$-amylase and/or disaccharidase digestion in the small intestine.

1.4 Resistant starch

Until the early 1980s, starch was thought to be completely hydrolyzed to glucose and the released glucose absorbed in the small intestine of man. However, some starches have been found to be partially resistant to pancreatic $\alpha$-amylase digestion in the small intestine. All starch and starch degradation products not absorbed in the small intestine of healthy individuals is termed resistant starch (RS) (EURESTA 1991).

Individuals vary widely in their ability to digest starch. What behaves as RS in one person, may not behave as RS in another. Therefore, there are no absolute distinctions between the RS and digestible starch in a particular starch sample (Thompson 2000b). For a given starch-containing sample, we can only determine the mean levels of RS for a population of individuals (Englyst et al. 1996).
1.4.1 Analytical procedures for RS determination

Because using a human population to monitor RS levels is inconvenient, much work has been done to develop in vitro procedures for determining RS (Berry 1986, Champ 1992, Englyst et al. 1992, Englyst and Macfarlane 1986, Faisant et al. 1995, Goñi et al. 1996, Htoon et al. 2009, McCleary and Monaghan 2002). The currently accepted procedure for the official in vitro determination of RS (AOAC Method 2002.02) was developed by McCleary and Monaghan (2002). After 16 hours digestion with pancreatic $\alpha$-amylase, the starch recovered by precipitation is determined. The 16 hours digestion time was validated by comparison of samples against in vivo RS as determined in human subjects (McCleary and Monaghan 2002).

Although the official in vitro procedure for determination of RS has been validated by comparison against in vivo RS, it is important to remember that the definition of RS is physiological. For a given RS-containing sample, we can only determine the mean levels of RS for a population of individuals (Englyst et al. 1996). Because RS is not a chemical entity, the amount and the nature of RS determined by any analytical procedure describes a putative RS. Nonetheless, the official in vitro determination of RS procedure is a convenient and reliable procedure for studying starch digestibility in vitro. In addition, by this method there is provision for recovery of undigested starch that should be reasonably representative of RS in vivo.

Rees (2008) has analyzed the kinetics of pancreatic $\alpha$-amylase digestion for some starch materials, including native CCS and HAMS granules. For native CCS granules, the rate of digestion becomes negligible well before 16 hours. For some other starches, including native HAMS granules, the rate of digestion did not fall to zero by 16 hours digestion. The kinetics of digestion for HAMS, shown in Fig. 1.5, suggested that HAMS would not be completely digested by pancreatic $\alpha$-amylase even by extending the digestion time beyond the stipulated 16 hours. This outcome suggests that the RS from HAMS may have a residual susceptibility to pancreatic $\alpha$-amylase digestion. The RS method development described by McCleary and Monaghan (2002) also supports this outcome.
Figure 1.5 Kinetics of pancreatic α-amylase digestion for HAMS granules. Figure adapted from Rees (2008).
1.4.3 Current methods to increase the RS content

Based on the reason for the enzyme resistance, a classification of RS has been established. Three types were originally described by Englyst et al. (1992): type 1, due to physical inaccessibility of the starch granules themselves, e.g. in whole grains or kernels; type 2, due to the inaccessibility of starch molecules in the raw starch granules from certain plants; type 3, due to the retrogradation, of starch molecules subsequent to heat treatment. Subsequently a type 4 was added to the classification. In type 4 the resistance is due to chemical modifications of the starch (Brown et al. 1995). These chemical modifications may possibly render the starch resistant to $\alpha$-amylase digestion. There may be a further category of RS (potentially type 5) which relies on the ability of the starch polymers, particularly amylose, and polar lipids to form inclusion complexes (Brown et al. 2006). These complexes may be useful in protecting the starch derived material during food processing and transit through the upper gastrointestinal tract (Brown et al. 2006). Although the classification of RS into these five types can be useful in understanding the basis for enzyme resistance, a starch sample could be resistant to enzymatic digestion for a number of these reasons, and a particular starch sample could include all five types of RS (Thompson 2000b).

A number of methods are available to enhance the RS content of starches. The most common preferred raw material for producing RS-containing ingredients is HAMS (Thompson 2000b). To enhance type 2 RS, the double helicity of granular starch is intentionally used as a means of driving further molecular organization, functioning as a sort of template. Enzyme resistance of granular starch is often enhanced by various combinations of time, temperature and moisture, referred to as hydrothermal treatments. Jacobs and Delcour (1998) have divided hydrothermal treatments into heat-moisture treatments (HMT), those performed at moisture levels below 35%, and annealing treatments, those performed at moisture levels greater or equal to 40%. By either type of hydrothermal treatment it is possible to enhance the RS level without destroying granular structure. Hydrothermal treatments are often used in combination with partial acid or enzyme hydrolysis (Brumovsky and Thompson 2001, Eerlingen et al. 1993, Sievert and Pomeranz 1989).
RS enhancement in non-granular starch, to produce type 3 RS, involves retrogradation of starch molecules subsequent to molecular dispersion of the native starch granules by heat and water, or by solvent. The most common method of dispersion is heating in water. In many common thermal treatments, it is likely that some residual molecular structure remains. Evans and Thompson (2008) hypothesized that the remaining molecular structures may constrain subsequent physical reassociation of chains and the formation of RS. In their study, dispersion in diluted sodium hydroxide was used to completely eliminate the ordered structures present initially (Evans and Thompson 2008). They observed RS in materials precipitated with either ethanol or ammonium sulfate. Liu and Thompson (2006) showed that rehydration after ethanol precipitation could lead to formation of RS from CCS when isoamylase was employed during rehydration.

The structures responsible for type 3 RS are thought to be based on associated double helices (Gidley et al. 1995). Eerlingen et al. (1993) found that the chain length of the material resistant to digestion (dp 19-26) for retrograded potato amylose was independent of the amylose average chain lengths originally used to form the type 3 RS; however, the yield of RS increased with the average chain length of the amylose originally used to produce it. Although type 3 RS is often attributed to amylose retrogradation (Eerlingen et al. 1993, Sievert and Pomeranz 1989), retrograded amylopectin has also been shown to contribute to type 3 RS (Eerlingen et al. 1994). This finding was also supported by Evans and Thompson (2008), who showed that both linear and branched fractions precipitated by solvent-dispersed HAMS seem to be involved in the development of crystallinity and helicity of the samples. Both the crystallinity and the helicity of the samples influenced the extent of RS. Berry (1986) showed that debranching of amylopectin is effective in generating RS from dispersed amylopectin.

One of the concerns about the properties of RS-containing starch materials generated is the thermal stability of the RS. For example, RS from retrograded amylopectin has limited practical utility, due to its sensitivity to loss by thermal treatments (Eerlingen et al. 1994). Several manufacturing processes have been developed to increase thermal stability of type 3 RS (Brumovsky and Thompson 2001, Chiu et al. 1994, Iyengar et al. 1991). In these processes, the preferred starch material was HAMS.
The only report about the production of thermally stable RS-containing materials from CCS was the work of Liu and Thompson (2006).

1.5 Fate of RS in the colon

Most starch entering the colon is further hydrolyzed there. It has been presumed that hydrolysis occurs by the amylolytic enzymes of the bacterial microflora of the large intestine. Only a small percentage, variable according to the amount of RS consumed, is excreted in the feces (Cummings et al. 1996). It has been estimated that 30-45 g of carbohydrates must be fermented each day to replace the bacterial cells lost in the feces (Tannock 1995). Microbes that degrade RS are important guests in the colon, as they may release carbohydrates that may be readily fermented by microbes that are not capable of utilizing RS.

The chemical and physical nature of the starch substrate is a critical factor in whether the starch will be hydrolyzed by pancreatic α-amylase in the small intestine. It is likely that those same properties also influence both hydrolysis rate and hydrolysis extent of RS in the colon. Residual susceptibility to pancreatic α-amylase amylolysis has been shown for some starch materials, including granular HAMS (Rees 2008). Thus, for these starch materials, the RS entering the colon is not completely resistant to pancreatic α-amylase digestion in the conditions of the small intestine, but the kinetics of digestion are sufficiently slow that the time required for complete hydrolysis exceeds the transit time in the small intestine. It does seem likely RS with greater residual susceptibility to hydrolysis may be more highly and readily available for microbial utilization in the colon, and that hydrolysis would be favored in proximal regions of the colon. Whether a higher and more rapid availability to microbial utilization for RS is a positive outcome is still to be understood. In fact, RS with a slower rate of degradation would be expected to be fermented in the more distal colonic region. Fermentation of the products of RS degradation in the distal colonic regions would be advantageous from a health standpoint, since those are the regions of the gut where the main cancerous disorders take place (Gibson et al. 2004).
The fermentation of RS in the colon leads to the production of hydrogen gas, carbon dioxide, methane and volatile fatty acids (SCFAs), mainly acetate, propionate, and butyrate. SCFAs induce a more acidic colonic environment. Greater acidity inhibits the growth of potentially pathogenic pH-sensitive organisms (Bird et al. 2000), and enhances the absorption of calcium and magnesium (Schulz et al. 1993). A low pH has also been linked to the precipitation of potentially toxic or carcinogenic compounds, such as secondary bile acids, diminishing their adsorption (Grubben et al. 2001). SCFAs are also important metabolic fuels for colonocytes, with butyrate being their preferred substrate (Bird et al. 2000). The presence of butyrate enhances growth of normal cells and inhibits that of malignant ones, by stimulating apoptosis of mutated cells (Hu et al. 2002, Le Leu et al. 2007, Topping and Clifton 2001). Overall, these observations have led to the hypothesis that RS may reduce the occurrence of colon cancer.

Although the consumption of RS may benefit colonic mucosal health, public health recommendations for RS intake have not been made at present. The dose at which physiological effects have been seen is of the order of 30 g daily or more (Kendall et al. 2004), but the RS intake in the current Western diet has been estimated in the order of 5-10 g/day (Brighenti et al. 1998). It appears simplistic to suppose that increased RS consumption will necessarily confer benefits to an individual. In fact, a substantial individual variation has been observed not only in the starch digestibility but also in the fermentability (Bird et al. 2000). In vitro data show that fecal inocula from some subjects are less capable of fermenting the products of starch hydrolysis than others (Cummings et al. 1996).

1.5.1 Microbiota in the human intestinal system

In a typical healthy human body microbes make up 1-2% of the body’s mass (Phillips 2008). The microorganisms that live in and on humans are known as the microbiota (Wilson 2004). The genomes of these microbial symbionts are collectively defined as the microbiome (Turnbaugh et al. 2007).

It has been estimated that the gastrointestinal tract (GIT) of an adult human harbors about $10^{14}$ viable cells, 10 times the total number of eukaryotic cells in all tissues
of the human body (Holzapfel et al. 1998). Most of the GIT microbiota are in the colon. Microbial cells make up about 55% of the colonic solid content (Tannock 1995).

Describing the composition of the colonic microbiota is a difficult task. Knowledge of the composition of the colonic microbiota of humans comes largely from bacterial culturing of feces. Studies of fecal specimens revealed that the colonic ecosystem is highly diverse. The colonic microbiota of healthy adults is comprised of at least 300-400 different culturable species belonging to more than 190 genera (Holzapfel et al. 1998). The numerically predominant bacteria are obligate anaerobes (Tannock 1995). The dominant genera, present at levels of at least $10^{11}$ cells/ml of colonic content or fecal material, are *Bacteroides, Eubacterium, Bifidobacterium* and *Peptostreptococcus* (Wilson 2004).

Several colonic bacterial groups, including *Eubacterium,* *Firmicutes,* *Bacteroides,* *Bifidobacterium,* and *Escherichia* can ferment starch (Brown et al. 1998, Macfarlane and Englyst 1986, Macfarlane and Macfarlane 1993). *Clostridium butyricum* and species of *Bifidobacterium* in particular can utilize HAMS efficiently *in vitro* (Brown et al. 1998). A mixed population of colonic bacteria (from a fecal slurry) completely hydrolyzed HAMS *in vitro* within 48 hours, suggesting that even the RS portion of HAMS can be degraded by fecal bacteria (Christl et al. 1997).

For their putative ability of influencing the ecology of the colonic microflora Brown et al. (1997) and Silvi et al. (1999) suggested that certain type of RS may act as prebiotics. A prebiotic can be defined as “a non-digestible food ingredient that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health” (Gibson and Roberfroid 1995). Since many of the colonic bacterial species are capable of utilizing starch, Gibson and Roberfroid (1995) do not consider RS as prebiotic, but as substrate for many microorganisms. *In vivo* studies in animals and humans on the selectively stimulation of the growth and/or activity of one or a limited number of bacteria in the colon provided by RS consumption are still inconclusive; therefore, the definition of RS as prebiotic is still arguable.

Several researchers have used *in vitro* procedures to investigate the question of which colonic bacterial species are capable of degrading starch (Bird et al. 2000,
Some have selected starch granules that have a high percentage of RS, such as granular HAMS and potato starch, but these starches also have a high proportion of digestible starch. In addition, most researchers have added these starch granules to a culture medium and then autoclaved the mixture (Crittenden et al. 2001, Ryan et al. 2006, Wang et al. 1999b), leading to a partial disruption of the granular structure, and consequently to a starch material that is in fact low in RS. For example, although granular HAMS is about 50% resistant, after autoclaving, it is only about 15% resistant (Wang et al. 1999b).

Moreover, a misuse of the term “resistant starch” is often encountered in the literature. In several publications (Bajka et al. 2007, Crittenden et al. 2001, Kleessen et al. 1997, Lesmes et al. 2008, Silvi et al. 1999, Wronkowska et al. 2006), to cite only few), the authors refer to resistant starch-containing materials as “resistant starch”. This situation is particularly confusing for high-amylose maize starch, which is often referred to (even by ingredient suppliers) as RS.

Only one study has been published where resistant material, putative RS, was obtained from the original RS-containing material. Putative RS from high-amylose maize starch or Actistar® (a retrograded long chain maltodextrin product obtained from tapioca starch) was utilized as substrate for in vitro fermentation, not by a particular microorganism but by human fecal material (Fässler et al. 2006). Moreover, the putative RS was obtained by two in vitro digestion assays that differ from the official in vitro determination of RS (AOAC Method 2002.02). Thus the crucial experiment to determine whether putative RS, that should be reasonably representative of RS in vivo, can be hydrolyzed and fermented by human fecal material or by a particular strain has not been yet performed.

Due to the lack of experimental data to determine whether a particular strain can hydrolyze RS in vitro, no information is available on whether differences in physical and chemical structures may lead to differences in RS microbial utilization and fermentability by a particular microorganism. However, in vivo studies seem to indicate that the structure of RS may play an important role in modulating the fermentation of RS in the colon. For example, Bird et al. (2007) fed a diet containing 50% native HAMS or heat-
moisture-treated HAMS (HMT) to young pigs. The chemical structure of these two starches is identical, but the order of the crystalline fraction (physical structure) is improved in the HMT as compared to in the native HAMS. In their study, Bird et al. (2007) showed that the effects of RS on in vivo fermentation from the HMT were more pronounced in the more distal region of the colon of young pigs as compared to native HAMS. Chemically modified starches have been shown to differ in their bifidogenic activity both in vivo (Brown et al. 2006, Wang et al. 2002) and in vitro (Brown et al. 1998). Although very likely, no evidence is yet available to support an effect of differences in starch chemical structure on RS microbial utilization and fermentability.

1.5.2 Bifidobacterium spp.

The bacteria of the genus Bifidobacterium are Gram positive bacilli, and are immobile and nonsporulate. The optimum temperature for growth is 37-41°C. Bifidobacteria are acid-tolerant microorganisms. The optimum pH is between 6.5 and 7.0 (Biavati et al. 2000). Bifidobacteria are all anaerobic, but the sensitivity to oxygen varies by the species, in some cases by strain (Ballongue 1998). In the genus Bifidobacterium, lactic and acetic acids (in theoretical final ratio 1.0:1.5) are produced as the major metabolic end products from hexose fermentation. Different species produce variable amounts of acetate, lactate, ethanol and formate (Ventura et al. 2004).

All Bifidobacterium species can be grouped in six different ecological niches: the human intestine, the oral cavity, food, the animal gastrointestinal tract and sewage (Ventura et al. 2004). The mayor habitat is considered to be the intestine of humans and other mammals. Twelve species have been associated with humans hosts (van den Broek et al. 2008), of which the most common are Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium infantis, Bifidobacterium adolescentis and Bifidobacterium brevis (Orrhage and Nord 2000). Bifidobacteria rapidly colonize the digestive tract of newly born infants. The number of known Bifidobacterium spp. present gradually decreases with age. For example, B. bifidum, B. catenulatum, B. pseudocatenulatum and B. longum biotype infantis are not found in elderly people (Hopkins and Macfarlane 2002, Hopkins et al. 2002).
Some bifidobacteria strains have several health-promoting effects for the host. A number of human studies suggest that bifidobacteria can decrease the incidence and prevent diarrhea, alleviate lactose intolerance and inhibit the activity of pathogens (Gibson and Roberfroid 1995, Parvez et al. 2006). Because increase in number of bifidobacteria in the colon is often associated with increased levels of butyrate in the colon, bifidobacteria are considered potentially helpful in decreasing the risk of colon cancer (Parvez et al. 2006). Curiously, bifidobacteria do not have the ability to produce butyrate themselves. It is likely that bifidobacteria are associated with increased butyrate levels due to their ability to hydrolyze RS outside the cell, increasing the level of readily metabolizable carbohydrate to be fermented by other anaerobic organisms, including butyrate-producers such as *Roseburia* sp (Belenguer et al. 2006).

While a number of *in vivo* studies investigated whether consumption of RS-containing diets may lead to proliferation of bifidobacteria in the colon, the question is yet to be answered. Increased proliferation of indigenous bifidobacteria and lactobacilli in rats fed a RS–based diet was observed by several groups (Kleessen et al. 1997, Silvi et al. 1999, Wang et al. 2002). Wang et al. (2002) also observed that the degree of stimulation varied depending on the type of starch fed to the rats. Although Bird et al. (2007) reported no increase in either lactobacilli or bifidobacterial numbers in the large bowel of young piglets fed a diet containing RS prepared from rice starch as compared to control diets containing highly digestible starch, the RS-containing diet was particularly effective in suppressing *E. coli*. Bouhnik et al. (2004) investigated in humans the bifidogenic property of a type 3 RS-containing material produced from debranched retrograded tapioca maltodextrin. Results were inconclusive.

Little is known about *Bifidobacterium* enzymes involved in carbohydrate breakdown. Van der Broek et al. (2008) suggested that bifidobacteria may be able to degrade starch to low molecular weight oligosaccharides by the action of extracellular amylolytic enzymes or of amylolytic enzymes present on the outer membrane of the bacterium. Subsequently, the oligosaccharides would be further degraded and internalized by an unknown mechanism. Extracellular amylolytic activity has been shown for *B. bifidum* and *B. pseudolongum* by Wang et al. (1999a) and for 11 other *Bifidobacterium* species by Ryan et al. (2006). To date, only three extracellular
Amylolytic enzymes have been isolated and purified from *Bifidobacterium* species (Ji et al. 1992, Lee et al. 1997, O’Connel Motherway et al. 2008). 

The questions of whether and how pure cultures of bifidobacteria strains can utilize RS are still open. Wang et al. (1999b) showed multiple amylolytic enzymes in extracts from *B. bifidum* and *B. pseudolongum*. They identified these enzymes as α-amylases on the SDS-PAGE pattern. However, identification of enzymes based on SDS-PAGE is not conclusive. The identification and recovery of only one α-amylase from *B. adolescentis* Int-57 has been published (Lee et al. 1997), but starch digestion has not been studied for this enzyme.

### 1.6 Project rationale

Starches may differ with respect to both digestion rate and digestion extent in the small intestine. The portion of starch not hydrolyzed by pancreatic α-amylase and absorbed in the small intestine is termed resistant starch (RS). The chemical and physical nature of the starch substrate is a critical factor in whether the starch will be digested by pancreatic α-amylase in the small intestine.

In the present study, novel RS-containing preparations will be produced by molecularly dispersing either common corn or high-amylose maize starches, precipitating the dispersed molecules, and rehydrating the precipitate in the presence of different levels of isoamylase. By varying the starch concentration and level of isoamylase at rehydration, RS-containing preparations with a range of enzyme susceptibility will be generated. The RS-containing preparations will be then chemically and physically characterized, with the purpose to relate enzyme susceptibility to the physical and chemical structure.

A number of methods are available to enhance the RS content of starches. However, only few have investigated the RS content of the RS-containing starch materials following a thermal treatment. HAMS is usually used as preferred raw material for manufacturing of RS-containing ingredients. Very little information is currently available on the methodology to produce thermally-stable RS-containing materials.
starting from CCS. In this study, thermally-stable RS starting from CCS and HAMS will be chemically characterized for both starch granules and starch preparations. This information is crucial in order to enhance the resistant starch content of starch-containing foods that are cooked prior consumption.

The official method for *in vitro* determination of RS (AOAC Method 2002.02) subjects the starch to 16 h digestion with pancreatic $\alpha$-amylase, and then the starch recovered by precipitation is determined. The 16 h digestion time has been validated by comparison of RS values against *in vivo* RS values as determined in human subjects. Analysis of the kinetics of complete digestion of pancreatic $\alpha$-amylase digestion for native CCS granules shows that the rate of digestion is negligible well before 16 h. However, for some other starches, including native HAMS granules, RS remains, and the rate of digestion does not fall to zero by the end of the 16 h digestion. The kinetic analysis suggests that the RS may have a residual susceptibility to pancreatic $\alpha$-amylase digestion (Rees 2008). There appear to be no information on the residual susceptibility to pancreatic $\alpha$-amylase digestion for RS from RS-containing starch preparations.

Resistant starches differ with respect to both hydrolysis rate and hydrolysis extent in the colon. It is generally assumed that hydrolysis in the colon is due to amylolytic enzymes of the bacterial microbiota. While a number of studies have used *in vitro* procedures to address the question of which colonic bacterial species are capable of degrading RS-containing materials, there appear to be no *in vitro* studies designed to address the question of which colonic bacterial species are capable of degrading putative RS obtained from a particular RS-containing material. Although bifidobacteria are believed to play an important role in the breakdown and fermentation of complex carbohydrates that reach the colon, no *in vitro* studies have been published investigating whether a particular bifidobacterium strain can hydrolyze putative RS *in vitro*. Due to the lack of experimental data, no information is available on whether the chemical and physical nature of RS may influence RS utilization and fermentability by a particular strain of *Bifidobacterium*.
Chapter 2: Overall goal, hypotheses and objectives

2.1. Overall goal of the PhD research

Produce and characterize resistant starch-containing preparations with different susceptibilities to pancreatic α-amylase, and compare the growth of selected strains of *Bifidobacterium* when prepared resistant starches with different susceptibility to further pancreatic α-amylase digestion are used as the main source of carbohydrate.

2.2 Thesis objectives and hypotheses

Objective 1: Produce and characterize novel thermally-stable resistant starch-containing preparations with a range of enzyme susceptibility from molecularly dispersed common corn and high-amylose maize starches.

Hypothesis 1a: Smaller particle size and a higher proportion of branched molecules lead to increased enzyme susceptibility of the resistant starch-containing preparations.

Hypothesis 1b: Helicity and crystallinity influence the extent of thermally-stable resistant starch and enzyme susceptibility of the resistant starch-containing preparations.

Objective 2: For selected novel resistant starch-containing preparations and selected commercial starches determine the time course of digestion by pancreatic α-amylase and the residual susceptibility of putative RS to continued pancreatic α-amylase digestion.

Hypothesis 2: Resistant starch from different starches or starch preparations varies in susceptibility to continued α-amylase digestion.
Objective 3: Compare growth of selected *Bifidobacterium* strains when prepared resistant starches with different susceptibility to further pancreatic α-amylase digestion are used as the main source of carbohydrate.

Hypothesis 3: The susceptibility to further α-amylase digestion influences the growth of *Bifidobacterium* strains when resistant starch is the main source of carbohydrate.
Chapter 3: Materials and methods

3.1 Materials

3.1.1 Chemicals

Common corn starch (CCS, Melojoy), high-amylose maize starch (HAMS, Hylon VII), and heat-moisture treated HAMS (HMT), were from National Starch and Chemical Co. (Bridgewater, NJ).

Pancreatic α-amylase (EC# 3.2.1.1) and amyloglucosidase (AMG, EC# 3.2.1.3) were from the Resistant Starch Determination Kit (Product# K-RSTAR) obtained from Megazyme International (Ireland). The isoamylase (EC 3.2.1.68) from Pseudomonas sp. was obtained from Megazyme International (Ireland). Activity reported was based on the activity determined by the manufacturer.

Ethanol (200 proof, Anhydrous, USP grade), acetone (ACS certified), and sodium hydroxide (ACS certified) were obtained from VWR Scientific Products (Bridgeport, NJ). Dimethyl sulfoxide (DMSO; Biology Grade) was obtained from Fisher Scientific (Fair Lawn, NJ).

3.1.2 Microbial strains

Strains of bifidobacteria (Table 3.1) were obtained courtesy of Dr. Robert F. Roberts who had obtained the strains designated ATCC from the American Type Culture Collection (Manassas, VA) and the strain designated with DSMZ from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), as well as from commercial suppliers. One commercial strain, designated BB had been obtained from Chr Hansen (Hørsholm, Denmark), and one designated Lafti had been obtained from DSM Food Specialties (Australia). Some commercial strains had been obtained previously in Dr. Roberts’ laboratory from six commercial starter culture suppliers and were designated as “RB” followed by a random four-digit number for identification purposes and maintained as frozen stocks at -70°C (Briczinski 2007).
Table 3.1 Strains of bifidobacteria utilized in the study

<table>
<thead>
<tr>
<th>Bifidobacteria strain</th>
<th>Species identity</th>
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<tr>
<td>ATCC 15703</td>
<td>B. adolescentis</td>
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<tr>
<td>ATCC 25527</td>
<td>B. animalis subsp. animalis</td>
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<tr>
<td>ATCC 27672</td>
<td>B. animalis subsp. animalis</td>
</tr>
<tr>
<td>ATCC 27536</td>
<td>B. animalis subsp. lactis lactis</td>
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<tr>
<td>Lafti B 94</td>
<td>B. animalis subsp. lactis lactis</td>
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<td>BB 12</td>
<td>B. animalis subsp. lactis lactis</td>
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<td>ATCC 25526</td>
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Prior to use in the experiments, a vial of the frozen stock culture from Dr. Roberts’ collection for each strain of bifidobacteria used in the current study was revived in 10 ml fresh MRS broth (de Man-Rogosa Sharpe; Difco, Becton, Dickson and Company, Sparks, MD) at 37°C for 24 h in an anaerobic chamber (Anaerobe Systems, Inc., San Jose, CA) with an atmosphere consisting of 10% carbon dioxide, 5% hydrogen and 85% nitrogen. Turbid cultures were then streaked for isolation on MRS agar and incubated at 37°C for 72 h in the anaerobic chamber. A single isolated colony from the agar plate was transferred to 10 ml MRS broth and incubated for 24 h at 37°C. Turbid broth was then mixed in equal amounts with 20% glycerol (w/v) (Fisher Scientific, Fairlawn, NJ). This suspension was transferred into sterile cryovials (Nalgene, Rochester, NY) and kept frozen at -70°C until needed.

Prior being used in the experiments, the strains were activated by transferring 200 μl of frozen stock turbid broth/glycerol suspension into 10 ml fresh MRS broth and incubating at 37°C for 24 h in the anaerobic chamber. Then 100 μl of the activated cultures was transferred into 10 ml fresh MRS broth and incubated at 37°C for 24 h in the anaerobic chamber. Between experiments, the cultures were maintained by transferring 100 μl of the cultures into 10 ml fresh MRS broth every 24 h and incubating at 37°C in the anaerobic chamber.

MRS medium normally contains 20 g/l added dextrose. For experiments in which dextrose-free MRS was used, the medium was prepared according to the formulation for MRS, but excluding the dextrose: 10 g/l bacto proteose peptone No. 3, 10 g/l bacto beef extract, 5.0 g/l bacto yeast extract, 1.0 g/l sorbitan monooleate complex, 2.0 g/l ammonium citrate, 5.0 g/l sodium acetate, 0.1 g/l magnesium sulfate, 0.05 g/l manganese sulfate, and 2.0 g/l disodium phosphate. Bacto proteose peptone No. 3, bacto beef extract, bacto yeast extract and sorbitan monooleate complex were obtained from Difco (Becton, Dickson and Company, Sparks, MD). Ammonium citrate, sodium acetate, magnesium sulfate, manganese sulfate, and disodium phosphate were obtained from VWR Scientific Products (Bridgeport, NJ). Agar plates were prepared by adding 15 g/l of bacto agar (BD, Franklin Lakes, NJ) to the broth prior autoclaving for 20 minutes at 121°C.
3.2 Generation of RS-containing starch preparations

3.2.1 Precipitation of dispersed starch

The scheme for the production of RS-containing starch materials from CCS and HAMS is shown in Fig. 3.1. This scheme is an elaboration of the conditions used in a procedure first employed by Liu and Thompson (2006), combining retrogradation with isoamylase treatment. At room temperature, starch dispersions in sodium hydroxide (0.5 N) were prepared at 1.0% (w/v) for common corn starch (CCS) and at 2.0% (w/v) for Hylon VII (HAMS) starch concentrations. Dispersion of CCS at 2.0% was attempted, but CCS could not be fully dispersed at this concentration.

CCS or HAMS were added to 50 ml of 0.5 N sodium hydroxide solution slowly and with constant stirring to avoid formation of clumps. The starches were dispersed in the sodium hydroxide under constant stirring at room temperature until the solution appeared clear (~2 hours), when the dispersion was considered complete. Four volumes of ethanol (v/v) were then added by rapid pouring into the dispersion. Immediately after the addition of ethanol, the samples were vigorously shaken, and then placed in a waterbath at 50°C and stored without disturbance. The precipitation was considered to be complete when the supernatant above the precipitate was clear. The ethanol precipitations were all complete in 2 hours.

The stir bars were removed, and the samples were centrifuged (B-20A Centrifuge; International Equipment Company; Needham, MA) at 1900 x g for 10 minutes. The supernatants were carefully decanted, and 50 ml of ethanol was added to the pellets. The samples were vigorously shaken and then centrifuged at 1900 x g for 10 minutes. This ethanol wash and centrifugation was repeated. The ethanol precipitates were then either dried or rehydrated.

The non-rehydrated samples (NO R) were dried in a convection oven at 50°C overnight. The rehydrated samples were rehydrated to achieve a 1% or 2% concentration (1%R, 2%R) by adding DI water slowly (initially drop by drop) and with constant stirring to avoid formation of clumps. The pH of the dispersion was adjusted to 5.0-5.1 with 1N and 0.1N HCl. The pH adjustment took ~30 min per sample.
Figure 3.1 Scheme for the production of RS-containing starch materials from CCS and HAMS.

IA= isoamylase; U= activity, expressed as units per mg of starch
For the samples rehydrated in the absence of isoamylase, stirring was ceased 30 min after the pH adjustment, and the samples were then stored at room temperature for 16 h without disturbance. For the samples rehydrated in the presence of isoamylase, 10 or 20 U of isoamylase/g starch was added to the samples immediately after the pH adjustment. Stirring continued for 30 min after the pH adjustment. Then the samples were then stored at room temperature for 16 h without disturbance.

The stir bars were removed, and the samples were centrifuged at 1900 x g for 10 min. The supernatants were carefully decanted. The precipitated starches were dried in a convection oven at 50ºC overnight.

The precipitates were weighed in order to calculate the starch yield prior to being ground using a mortar and pestle and passed through a sieve (#80, <180 μm mesh). Starch yield was calculated as:

\[
Starch\ yield\ (%) = \frac{weight_{\text{precipitate\ (dry\ matter)}}}{weight_{\text{starch\ (dry\ matter)}}} \times 100
\]

The weight of the starch was measured prior to dispersion in sodium hydroxide. Three replicates preparations per each RS-containing starch material were analyzed.

### 3.2.2 Generation of RS-containing starch materials with different particle size

CCS 2%R-20U and HAMS 2%R-20U were prepared as described in 3.2.1.

The dried starch materials were ground using a mortar and pestle and passed through a series of mesh sieves stacked on each other in the following order (from top to bottom): sieve #35 (particle size of the sieved particles <500 μm), #45 (particle size of the sieved particles <355 μm), #60 (particle size of the sieved particles <250 μm), #80 (particle size of the sieved particles <180 μm), and #120 (particle size of the sieved particles <125 μm). This system allowed separation of ground particles into 5 fractions, according to their particle size: 355<x<500 μm, 250<x<355 μm, 180<x<250 μm, 180<x<125 μm, and <125 μm.
3.3 Analytical procedures for resistant starch analysis

3.3.1 Official method for \textit{in vitro} RS determination

The official method for \textit{in vitro} resistant starch (RS) determination (AOAC 2002.02, AACC 32-40) was employed, as scaled-down by Evans and Thompson (2008). The modification allows analysis of RS content for small starch samples (~20 mg instead of 100 mg).

Approximately 20 mg starch was weighed into 50 ml polypropylene centrifuge tubes. 4 ml of enzyme buffer was added to the sample. The sample was vortexed and immediately placed into a shaking waterbath (200 strokes/min) at 37ºC.

The enzyme buffer was prepared daily immediately before use. Pancreatic \( \alpha \)-amylase (2 mg/ml, 3 Ceralpha Units/mg) was dissolved in sodium maleate buffer (0.1 M, pH 6, containing 0.3 g/l CaCl\(_2\) and 0.2 g/l NaN\(_3\)) by stirring for 5 min. Amyloglucosidase (2 μl/ml, 3300 U/mL) was added and the suspension was stirred for 1 min. The dispersion was then centrifuged at 1,500 x g for 10 min (B-20A Centrifuge; International Equipment Company; Needham, MA). The supernatant was used as enzyme buffer.

After 16 h of digestion, the tube was removed from the waterbath and 4 ml of ethanol was added. The tube was vortexed and centrifuged at 1,500 x g for 10 min. The supernatant was removed and the precipitates were washed with 4 ml of 50% ethanol (v/v). The tube was centrifuged again at 1,500 x g for 10 minutes. The supernatant was decanted and the washing step was repeated. The supernatant was again carefully removed. The pellet was dispersed in 2 ml of 2 M potassium hydroxide with stirring for 20 min in an ice/water bath. Then 8 ml of 1.2 M sodium acetate buffer (pH 3.8) was added to each tube with stirring. Immediately a 0.1 ml aliquot of amyloglucosidase (AMG; 3300 U/ml) was added and the tube placed in a waterbath at 50ºC for 30 min, and vortexed every 10 min.

A 0.1 ml aliquot was added to small test tubes containing 3 ml of GODOP reagent (glucose oxidase > 12000 U/l, peroxidase > 650 U/l, 4-aminoantipyrine 0.4 mM) and incubated at 50ºC for 20 min. The absorbance of the samples was read at 510 nm against a reagent blank and compared to the glucose standard supplied with the kit. The proportion of precipitated starch, which in this method is considered to be the proportion
of RS, was calculated as:

\[
%\ RS = \frac{Glucose\ (mg)}{Dry\ weight\ of\ the\ samples\ (mg)} \times 0.9 \times 100
\]

Three replicates per each RS-containing starch material were analyzed. The mean of two starch samples analyzed on the same day was considered one replicate. Each replicate was obtained on a different day to account for possible day-to-day variations. A standard deviation was reported for all determinations.

3.3.2 Determination of RS after thermal treatment

The RS content after thermal treatment was determined by adding 2 ml of sodium maleate buffer (0.1 M, pH 6, containing 0.3 g/l CaCl₂ and 0.2 g/l NaN₃) to approximately 20 mg starch. The sample was vortexed and immediately placed into a boiling waterbath, for 30 min, and vortexed every 10 min. The sample was then cooled in ice for 5 min, before adding 2 ml of concentrated enzyme buffer (pancreatic α-amylase, 4 mg/ml, 3 Ceralpha Units/mg; amyloglucosidase 4 μl/ml, 3300 U/mL; in sodium maleate buffer) prepared daily immediately before use. Then the RS content was determined as described in 3.3.1.

3.3.3 Kinetic analysis of digestion with the RS assay

3.3.3.1 Sampling and chemical analysis over time

The time course of digestion was constructed by modifying the official in vitro RS determination method (AOAC Method 2002.0) as in Evans and Thompson (2008) and extending the digestion time from 16 to 48 h.

Aliquots (200 μl) of sample were removed at different time intervals (1 min, 5 min, 10 min, 15 min, 30 min, 1 h, 2 h, 3 h, 5 h, 8 h, 12 h, 16 h, 24 h, 32 h, and 48 h), and added to 95% ethanol containing 0.5% EDTA (1:1 v/v). After centrifugation (1,500 x g for 10 min), the supernatant was analyzed in duplicate for total carbohydrate using the phenol sulfuric acid method (Dubois et al. 1956). The percent digested starch was
calculated from this data and used as the basis for the calculation of the non-digested starch value as:

% non-digested starch = 100 – \[\frac{\text{Digested starch (mg)}}{\text{Dry weight of the sample (mg)}} \times 100\]

RS was determined from the aliquot removed at 16 h of digestion.

All samples were analyzed in triplicate. Each replicate was obtained on different days to account for possible day-to-day variations.

3.3.3.2 Digestion time-course analysis

The digestion time course data were first analyzed following the method developed by Rees (2008). A “Double, 5 parameter” regression model in SigmaPlot 8.0 (SRSS Inc., Chicago, IL) was used to fit the data using the double exponential decay equation:

\[S_t = S_1 e^{-k_1 t} + S_2 e^{-k_2 t} + S_{ND}\]  \hspace{1cm} (Eq. 1)

were \(S_t\) is the percentage of undigested starch, \(t\) is the time, \(S_1\) and \(S_2\) are the percentage of two distinct starch substrates, and \(k_1\) and \(k_2\) are the reaction rate constants for the decay of substrates \(S_1\) and \(S_2\). \(S_{ND}\) corresponds to the percentage of starch not digested by pancreatic \(\alpha\)-amylase.

For some samples, the double exponential decay model did not seem to fit the experimental data points collected at 24, 32 and 48 h. Therefore, for all samples a “Triple, 6 parameter” regression model in SigmaPlot 8.0 was selected to fit the data using the triple exponential decay equation:

\[S_t = S_{1a} e^{-k_{1a} t} + S_1 e^{-k_1 t} + S_2 e^{-k_2 t}\]  \hspace{1cm} (Eq. 2)

where \(S_t\) is the percentage of undigested starch, \(t\) is the time, \(S_{1a}\), \(S_1\) and \(S_2\) are the percentage of three distinct starch substrates, and \(k_{1a}\), \(k_1\) and \(k_2\) are the reaction rate constants for the decay of substrates \(S_{1a}\), \(S_1\) and \(S_2\).
A Fisher F-test for best fit was performed to compare the fit of the double (5 parameter) exponential decay model and the fit of the triple exponential (6 parameter) decay model. The F-value was calculated as:

\[
F\text{-value} = \left( \frac{SS_1 - SS_2}{SS_2} \right) / \left( \frac{(DF_1 - DF_2)}{DF_2} \right)
\]  
(Eq. 3)

where \(SS_1\) is the sum of squares of the residuals of the simpler model (in this case, the double exponential decay model), \(SS_2\) is the sum of squares of the residuals of the more complex model (in this case, the triple exponential decay model), \(DF_1\) is the degree of freedom of the simpler model (in this case, the double exponential decay model), and \(DF_2\) is the degree of freedom of more complex model (in this case, the triple exponential decay model). The F-value was then used to calculate the p-value, by using “The Free Statistics Calculators Website” (Soper 2009). If the p-value was < 0.05, the triple exponential decay model (Eq. 2) was considered a better model to fit the data as compared to the double exponential decay model (Eq. 1).

For samples for which the triple exponential decay model was considered to be a better model to fit the data, a “Triple, 7 parameter” regression model in SigmaPlot 8.0 was also selected to fit the data using the triple exponential decay equation:

\[
S_t = S_{1a} e^{-k_{1a} t} + S_1 e^{-k_1 t} + S_2 e^{-k_2 t} + S_{ND}
\]  
(Eq. 4)

where \(S_t\) is the percentage of undigested starch, \(t\) is the time, \(S_{1a}, S_1\) and \(S_2\) are the percentage of three distinct starch substrates, and \(k_{1a}, k_1\) and \(k_2\) are the reaction rate constants for the decay of substrates \(S_{1a}, S_1\) and \(S_2\). \(S_{ND}\) corresponds to the percentage of starch not digested by pancreatic \(\alpha\)-amylase.

A Fisher F-test for best fit was performed to compare the fit of the two triple exponential decay models (Eq. 2 and 4). If the p-value was < 0.05, the triple exponential decay model described by Eq. 4 would have been considered a better model to fit the data as compared to the triple exponential decay model described by Eq. 2.
3.3.4 Recovery of starch resistant to 16 hour digestion

3.3.4.1 Recovery of starch resistant to 16 hour digestion by the official method for \textit{in vitro} RS determination

Starch samples were digested as described in the official method for \textit{in vitro} RS determination (section 3.3.1). The isolation of starch resistant to 16 h digestion followed the Megazyme RS assay procedure (Cat. No. K-RSTAR; Megazyme, Ireland), and was designated ERS. After 16 h, the tube was removed from the waterbath, and 1 volume of ethanol was added to the sample and vigorously vortexed. The mixture was then centrifuged at 1,500 x g for 10 min. The supernatant was carefully decanted; the pellet was resuspended in 8 ml of 50% ethanol with vigorous vortexing, and centrifuged at 1,500 x g for 10 min. This suspension and precipitation step was repeated once more, and the pellets were air dried at room temperature for 24 h.

3.3.4.2 Recovery of starch resistant to 16 hour digestion by modification of the official method for \textit{in vitro} RS determination

Starch samples were digested as described in the official method for \textit{in vitro} RS determination (section 3.3.1). The starch resistant to 16 h digestion was isolated by modifying the Megazyme RS assay procedure (Cat. No. K-RSTAR; Megazyme, Ireland), and was designated ERS\textsubscript{L-AMY}. After 16 h of digestion, the tube was removed from the waterbath, and directly centrifuged at 1,500 x g for 10 min without addition of ethanol. The supernatant was carefully decanted; the pellet was resuspended in sodium maleate buffer (instead of 50% ethanol) with vigorous vortexing, and centrifuged at 1,500 x g for 10 min. This suspension and precipitation step was repeated once more, and the pellets were air dried at room temperature for 24 h.

3.3.5 Further digestion of the recovered ERS

ERS\textsubscript{L-AMY} from HAMS, HMT, HAMS 2\%R-20U, and CCS 2\%R-20U (prepared as described in 3.2.1) was resuspended in either 4 ml of sodium maleate buffer (0.1 M, pH 6, containing 0.3 g/l CaCl\textsubscript{2} and 0.2 g/l NaN\textsubscript{3}) or 4 ml of enzyme buffer (as prepared in
3.8.1.2) with vigorous vortexing. The sample was immediately placed into a shaking waterbath (200 strokes/min) at 37°C. Aliquots (200 µl) of sample were removed at different time intervals (1 h, 3 h, 6 h, 14 h, 24 h, and 48 h), and added to 95% ethanol containing 0.5% EDTA (1:1 v/v). After centrifugation (1,500 x g for 10 minutes), the supernatant was analyzed in duplicate for total carbohydrate using the phenol sulfuric acid method (Dubois et al., 1956). The percent digested starch was calculated from this data and used as basis for the calculation of the non-digested starch value.

All samples were analyzed in duplicate. The mean of two samples analyzed on the same day was considered one replicate. Each replicate was obtained on a different day to account for possible day-to-day variations.

3.4 Chemical characterization of RS-containing preparations

3.4.1 Size exclusion chromatography

A replicate of each RS-containing starch preparation was analyzed by chromatography on a Sepharose CL-2B column using gravity flow according to the procedures in Klucinec and Thompson (1998). The mobile phase in the system was 0.01 M sodium hydroxide containing 0.02% (w/v) sodium azide.

Starch samples (15 mg) were dispersed in 1 ml of 90% DMSO at room temperature under constant stirring overnight. The DMSO/starch dispersion was then diluted with 5 ml mobile phase, and then loaded onto the column using a sample applicator. The flow rate was adjusted to 20-30 ml/hr. For each sample, 500 ml of eluent was collected as 5 ml fractions using a fraction collector. Total carbohydrate and iodine binding analysis of fractions followed the procedure described in Klucinec and Thompson (1998). The void volume and the salt volume of the column were determined using a mixture of 1 mg of waxy corn starch and 1 mg of glucose.

3.4.2 High-performance size exclusion chromatography

High-performance size exclusion chromatography analysis of debranched starch samples was done by the method of Klucinec and Thompson (1998) with slight
modifications. Except for the replacement of a new injector (Model 7725i; Rehodyne) the HPSEC system and the conditions of the separation were the same as those previously used (Klucinec and Thompson, 1998). Debranching of starch samples followed the procedures in Klucinec and Thompson (1998). In addition, following debranching, samples were desalted and subjected to HPSEC analysis as described in Xia (2005).

To construct a trinomial standard curve, maltose (DP 2), maltotriose (DP 3), maltoheptaose (DP 4), and three pullulan standards, P-5 (MW 5.8x10^3, ~DP 36), P-10 (1.22x10^4, ~DP 75), and P-50 (MW 4.80x10^4, ~DP 296) were used.

Two independent debranching experiments were conducted for two replicates of each RS-containing starch preparations.

3.5 Physical characterization of selected RS-containing preparations

3.5.1 Wide angle X-ray diffraction

Wide angle X-ray diffraction (WXRD) analyses were conducted using a Rigaku MiniFlex II desktop X-ray diffractometer (Rigaku Americas Corporation, TX). Samples were equilibrated for at least 10 days over saturated magnesium nitrate solution (conditions of 55% relative humidity) prior to analysis. The moisture content of the samples was determined for each sample, and was between 12-15% (data not shown) after equilibration.

A small amount of sample (~ 20 mg) was compressed within two stainless steal screws, in order to obtain a circular pellet of ~ 3 cm diameter. The pellet was then loaded at the center of a zero-background sample holder and analyzed. Sample were analyzed between 4º and 32º 2Θ at a step size of 0.02º, a scan speed of 0.02º/ min, at a tension of 30 kV, and a current of 15 mA. The WXRD data was analyzed and plotted using MDI Jade 8 XRD pattern processing and identification software (MDI Materials Data, St. Louis, MO). Two replicates of CCS, HAMS, CCS NO R, HAMS NO R, CCS 2%R, HAMS 2%R, CCS 2%R-20U, and HAMS 2%R-20U were analyzed. The particle size of the RS-containing starch preparations was <180 μm.
Figure 3.2 Illustration of a) the total area above the baseline; and b) the area of the crystalline portion used to calculate the proportion of crystallinity for the RS-containing preparations obtained from CCS and HAMS.

Sample shown: HAMS (other samples are shown in Appendix A). Percent crystallinity was calculated as: \( \% \text{ crystallinity} = \left( \frac{\text{crystalline area}}{\text{total area}} \right) \times 100 \)
For CCS, HAMS, CCS 2%R-20U, and HAMS 2%R-20U, the area of the crystalline portion was estimated as in Evans (2005) by connecting the base of the peaks in the diffractograms, as illustrated in Fig. 3.2. The proportion of crystallinity was calculated by dividing the area of the crystalline portion by the total area above the baseline.

### 3.5.2 Solid-state $^{13}$C CP / MAS NMR

Prior to analysis by $^{13}$C CP/MAS NMR the samples were equilibrated for at least 10 days over saturated magnesium nitrate solution (conditions of 55% relative humidity). The moisture content of the samples was between 12-15% (data not shown) after equilibration. Samples were analyzed using a solid-state NMR spectrometer (CMX-300, Chemagnetics) operating at 74.8 MHz. Samples were packed into a 7 mm magic angle spinning (m.a.s.) rotor and spun at 3.5KHz. The 90º pulse width was 3.75 μs with a recycle time of 2 s. A contact time of 1 ms was used for all samples. Spectral width was 30 KHz; acquisition time, 17 ms. Granular WCS was analyzed immediately after analysis of the reference standard. This sample was used to correct the ppm values for other samples due to minor variations in the magnetic field. Spectra were adjusted slightly in order to align the peak maxima of the main peak to the main peak of granular WCS, at 73.2 ppm.

The NMR data were analyzed using the peak fitting function of Origin Software (Version 8.1; Originlab, Massachusetts, USA). A Gaussian shape was assumed for all peaks. The carbons of the glucose monomers of starch have been assigned to specific positions in the NMR spectra with C1 = 101-103 ppm, C2, C3, C5 = a broad peak in the region 67-75 ppm, C4 = 82 ppm, and C6 = 63 ppm. The peaks at 82 and 103 ppm have been associated to single helices (Paris et al. 1999, 2001). The peak at 101 ppm has been associated with double helices (Gidley and Bociek 1985). Signal intensity at 94-98 ppm for crystallized branched material has been assigned to amorphous character (Gidley and Bociek 1985). Resonances in the 97-98.6 ppm ranges have been shown to be influenced by the starch samples preparation procedure (Paris et al. 2001). For all samples, 5 peaks were fitted. Four peaks were fitted with a peak maximum set at the maximum of
individual peaks using the Origin Software. The fifth peak was fitted at 97 ppm.

The amorphous (as defined by X-ray diffraction, Fig. 3.3) reference was prepared by dispersing waxy maize starch in 0.5 N sodium hydroxide (2% w/v) at room temperature, under constant stirring overnight. After dispersion, four volumes of ethanol were rapidly added. The sample was vigorously shaken, and centrifuged at 1900x g for 10 minutes. The supernatant was carefully decanted, and the precipitate was washed with ethanol twice, before being dried in a forced air oven at 50°C overnight.

3.5.3 Differential scanning calorimetry

Approximately 15 mg of starch (as is basis) was weighed in a stainless steel DSC pan (product number 319-1605; Perkin-Elmer; Waltham, Massachusetts) and brought to approximately 33% solids by the addition of deionized water. The sample was stirred using a clean needle to avoid formation of clumps. The pan was sealed with the gasketed stainless steel lid and stored at room temperature.

Two replicates of CCS, HAMS, CCS NO R, HAMS NO R, CCS 2%R, HAMS 2%R, CCS 2%R-20U, and HAMS 2%R-20U were analyzed in the differential scanning calorimeter. The particle size of the RS-containing starch preparations was <180 μm. Before analysis, all samples were held at room temperature for ~24 h, with exception of the NO R samples, which were held for only 30 minutes.

The pan was placed in a differential scanning calorimeter (DSC-7 Perkin-Elmer; Waltham, Massachusetts), and subjected to a temperature profile as follows: holding the sample at 5°C for 1 min, heating from 5°C to 180°C at 10°C/min, cooling from 180°C to 5°C at 10°C/min, and re-heating from 5°C to 180°C at 10°C/min. An empty DSC pan was used as a reference. Temperature and enthalpy calibrations were based on an indium standard.

Data analysis was performed with the Perkin-Elmer Data Analysis software and repeated in triplicate for each starch material.
Figure 3.3 Wide angle X-ray diffraction of the ethanol precipitate prepared from waxy maize starch dispersed in sodium hydroxide.
3.6 Chemical characterization of ERS from the RS-containing preparations

ERS from the RS-containing starch materials was dispersed by boiling in 90% DMSO (starch concentration 41.7 mg/ml) for 10 minutes. 120 µl of the dispersion was then added to 880 µl sodium acetate buffer (pH 3.75, 0.05 N) at 50°C. Samples were then debranched as described in 3.4.2.

3.7 Agar plate assays

3.7.1 Determination of the zone on agar plate assays

3.7.1.1 Soluble-starch agar plate assay

Three *Bifidobacterium* strains (*B. pseudolongum* ATCC 25526, *B. animalis ssp. lactis* Lafti B94 and *B. animalis ssp. lactis* DSMZ 10140) were grown in MRS broth at 37°C for 24 h in an anaerobic chamber (as described in 3.1.2). The optical density at 600 nm at the end of the 24 h period, was measured to be > 0.6 for all three strains.

A small aliquot of the culture was transferred to an agar plate, by either transferring 10 or 15 µl of the broth to a sterile paper discs (6 mm) placed on the surface of the agar plates (as in Wang et al., 1999), or by transferring 5, 10 or 15 µl of the broth to an individual well (diameter ~7mm) formed in the agar plate (as in Ryan et al., 2006) by the tip of a sterile 15 ml disposable pipette. Soluble starch (5 g/l) replaced dextrose in the MRS agar (Section 3.1.2) as the main carbohydrate source. The soluble starch was added to the other ingredients prior to autoclaving the medium. The inoculated plates were incubated in an anaerobic chamber at 37°C for 2 days.

Following incubation, 5 ml of 0.15% iodine solution (I₂:KI=1:10) was transferred to the surface of the plate. For some strains, unstained zones around the wells were visualized as compared to the dark blue background of the iodine stained plate. For cultures inoculated using the disk, the distance between the border of the zone and the disk was measured with a ruler. For cultures inoculated in the wells, the radius of the
zone (from the border of the wells) was measured with a ruler. Although most of the clearing zones were circular, some of them had a less symmetric shape. Therefore, two measurements were taken per each zone: one measurement was taken on the longest diameter of the zone, and the second measurement was taken on the shorter diameter of the zone.

### 3.7.1.2 HAMS or ERS from HAMS agar plate assays

HAMS or ERS from HAMS (5 g/l) replaced the dextrose in the MRS agar (Section 3.1.2) as the main carbohydrate source. HAMS and ERS from HAMS were added subsequent to autoclaving and cooling the medium to 50°C (resulting in granular starch in the medium) prior pouring the plates. To minimize microbial contamination, the starch sample was washed with 80% ethanol and exposed to UV light for 30 min before addition to the medium. The starch sample was added to the medium slowly and with constant stirring to avoid formation of clumps.

10 µl of the culture described in 3.6.1.1 was transferred into a well on the native HAMS-containing agar plate. The plates were then incubated in an anaerobic chamber at 37°C for 4 days. The extent of starch degradation was estimated by measuring the radius of the light zones around the wells, as visualized by iodine staining. The iodine staining was done either by transferring 5 ml of 0.15% iodine solution (I₂:KI=1:10) to the surface of the plates or by exposing the plates to iodine vapor that generated from ~2 g of iodine crystals over 30 min at room temperature.

### 3.7.2 Agar plate assays for pancreatic α-amylase solution

A pancreatic α-amylase solution (0.05 mg/ml, 25 U/mg) was prepared by adding the enzyme to autoclaved MRS broth. Sterile laboratory supplies and gloves were used throughout the preparation of the pancreatic α-amylase solution in order to minimize possible microbial contamination. Serial dilutions of the pancreatic α-amylase solution were made, and 10 µl of each dilution was transferred to an individual well in a prepared soluble starch or HAMS-containing agar plate. The agar plates were then incubated in an
anaerobic chamber at 37°C for 4 days. Following incubation, the plates were stained with iodine and the radius of the zones was measured.

3.7.3 Agar plate assays for strains of *Bifidobacterium*

The 37 strains of *Bifidobacterium* in Table 3.1 were tested to evaluate the extent of extracellular amylolytic activity on soluble starch and granular HAMS. The strains were grown in MRS broth as described in 3.6.1.1. 10 µl of the culture was transferred to a well on a soluble starch or HAMS-containing agar plate, prepared as described in 3.6.1.1; and 3.6.1.2, respectively. The plates were incubated in an anaerobic chamber at 37°C for 4 days. The extent of starch degradation was estimated by measuring the radius of the light zones around the wells as visualized by iodine staining. The iodine staining was done by transferring 5 ml of 0.15% iodine solution (I$_2$:KI=1:10) to the surface of the soluble starch-containing plates, or by exposing the HAMS-containing agar plates to iodine vapor for 30 min.

All strains were analyzed in triplicate. The mean of the radii of two zones on soluble starch or HAMS-containing agar plate per strain analyzed on the same day was considered one replicate. Each replicate was obtained in different days to account for possible day-to-day variations. A standard deviation was reported for all samples.
3.8 Estimation of soluble amylolytic enzyme activity secreted by selected strains of *Bifidobacterium* in presence of different carbohydrate substrates

The scheme for the assay used for the estimation of soluble amylolytic enzyme activity secreted by selected strains of *Bifidobacterium* in presence of different carbohydrate substrate is shown in Fig. 3.4. 80 μl of the grown culture of six *Bifidobacterium* strains (*B. animalis* ssp. *lactis* BB12, *B. bifidum* RB 3046, *B. choerinum* ATCC 27686, *B. infantis* ATCC 15697, *B. animalis* ssp. *lactis* DSMZ 10140, and *B. pseudolongum* ATCC 25526) was transferred to 8 ml of either MRS broth or a prepared test-starch-containing broth with 0.5% soluble starch, granular HAMS or ERS from HAMS as the main carbohydrate source. Dextrose-free MRS broth (Section 3.1.1) was prepared and either soluble starch, HAMS or RS from HAMS (5 g/l) was added as main carbohydrate source. Soluble starch was added to the other ingredients prior autoclaving the medium. HAMS and ERS from HAMS were washed with 80% ethanol and exposed to UV light for 30 min and added subsequent to autoclaving and cooling the medium to 50°C. The inoculated media were incubated in an anaerobic chamber at 37°C for 24 hours.

After incubation, cell-free supernatants (CFS) were obtained by centrifuging 1.5 ml growth medium at 18,000 g for 2 min and subsequently filtering the supernatant (pore diameter 0.45 μm) to remove any residual cells or insoluble starch. CFS from the uninoculated test-starch-containing broth was used as negative control. An aliquot (10 μl) of the CFS was transferred to a well in a soluble starch-containing agar plate (prepared as in 3.6.1.1). Inoculated plates were then incubated in an anaerobic chamber at 37°C for 4 days, stained with iodine solution and the unstained zones were measured with a ruler.

In a second experiment, after the 24 h incubation, the CFS were concentrated by using a Centricon YM10 (Amicon, Beverly, MA) at 4°C for 1 hour at 7000 g. An aliquot (10 μl) of the concentrated CFS was transferred to a well in a soluble starch-containing agar plate. Following 4 days incubation in an anaerobic chamber at 37°C, the plates were stained with an iodine solution, and the radius of the unstained zones was measured.

All concentrated CFS were analyzed only once.
Figure 3.4 Assay for the estimation of soluble amylolytic enzyme activity secreted by selected strains of *Bifidobacterium* in presence of different carbohydrate substrates
3.9 Residual amylolytic activity associated with starch recovered after 16 h digestion

3.9.1 Evaluation of the extent of residual amylolytic activity

The scheme for the assay used for the estimation of the extent of residual amylolytic activity associated with the starch recovered after 16 h digestion is shown in Fig. 3.5. ERS and ERS\textsubscript{L-AMY} were washed with 80% ethanol and exposed to UV light for 30 min and then added to autoclaved MRS broth (described in section 3.1.1) at a final concentration of 5 g/l. The ERS-containing and ERS\textsubscript{L-AMY}-containing MRS broth was kept at room temperature for 1 h, prior being vortexed, and while starch was still suspended, an aliquot (10 μl) was transferred to an individual well in a prepared soluble starch-containing agar plate (prepared as in 3.6.1.1).

Starch-free supernatants (SFS) were obtained by centrifuging 1.5 ml of ERS\textsubscript{L-AMY}-containing MRS broth at 16,000 g for 2 min and subsequently filtering the supernatant (pore diameter 0.45 μm) to remove any residual insoluble starch. SFS from HAMS-containing MRS broth was used as negative control. An aliquot (10 μl) of the SFS was transferred to an individual well in a prepared soluble starch-containing agar plate.

The soluble starch-containing agar plates were then incubated in an anaerobic chamber at 37°C for 4 days. Following incubation, the unstained zones visualized using an iodine solution were measured with a ruler.

All starch samples were analyzed in triplicate. The mean of the radii of two zones on soluble starch-containing agar plate per starch sample analyzed on the same day was considered one replicate. Each replicate was obtained in different days to account for possible day-to-day variations. A standard deviation was reported for all samples.
Figure 3.5 Assay for the estimation of the extent of residual amylolytic activity associated with the starch recovered after 16 h digestion
3.9.2 Reducing the residual amylolytic activity associated with starch resistant to 16 h digestion

3.9.2.1 Resuspension in different solutions

ERS\textsubscript{L-AMY} from HAMS was resuspended in three solutions: 1) potassium thiocyanate solution, chosen because the highest solubility of pancreatic $\alpha$-amylase is in presence of thiocyanate ions (Faber et al. 2008); 2) EDTA solution, which inactivates pancreatic $\alpha$-amylase by dissociating the $\text{Ca}^{2+}$ from the active enzyme molecule causing enzyme denaturation (Lecker and Khan 1996, 1998); 3) SDS solution, which disrupts the non-covalent bonds in the proteins, causing protein denaturation. This surfactant was found to not cause cold gelatinization in starch granules (Seguchi and Yamada 1989).

ERS\textsubscript{L-AMY} from HAMS was resuspended in 4 ml of potassium thiocyanate (0.4 M), EDTA (0.1%), or SDS (0.1%) solutions and kept at room temperature for 30 min under constant stirring. Then the samples were centrifuged at 1,500 g for 10 min. The precipitates were then resuspended in 8 ml of potassium thiocyanate (0.4 M) and centrifuged at 1,500 g for 10 min. The resuspension and centrifugation step was repeated once. These last precipitates were washed with 80% ethanol and exposed to UV light for 30 min. The extent of residual amylolytic activity was estimated by using the methodology in 3.8.1.

3.9.2.2 Extended EDTA treatment

ERS\textsubscript{L-AMY} was resuspended in a 0.1% EDTA solution (12.5 mg/ml) with vigorous vortexing. The sample was immediately placed into a shaking waterbath (200 strokes/min) at 37ºC as in Lecker and Khan (1998). Aliquots (200 $\mu$l) of sample were removed at different time intervals (1 h, 2 h, 4 h, 8 h, 14 h, and 24 h), and centrifuged at 1,500 x g for 10 min. The sample obtained at 24 h was designated ERS\textsubscript{VL-AMY}. The precipitates were washed with 80% ethanol and exposed to UV light for 30 min. The extent of residual amylolytic activity was estimated by using the methodology in 3.8.1. The scheme for the assay used for the estimation of the extent of residual amylolytic activity associated with ERS\textsubscript{VL-AMY} is shown in Fig. 3.6.
ERS\textsubscript{VL-AMY}

- Wash with 80\% ethanol
- UV light, 30 min
- Added to autoclaved MRS broth (0.5\%)
- Room temperature for 1 h
- 10 \( \mu l \) unfiltered broth

Anaerobic incubation 4 day, 37\(^\circ\)C

Iodine staining

The radius of the unstained zone was measured

**Figure 3.6** Assay for the preparation and estimation of the extent of residual amylolytic activity associated with ERS\textsubscript{VL-AMY}. 
3.10 Growth of *Bifidobacterium* strains on starch resistant to 16h digestion

3.10.1 Growth on ERS<sub>L-AMY</sub> or ERS<sub>VL-AMY</sub> from HAMS

Three *Bifidobacterium* strains (*B. pseudolongum* ATCC 25526, *B. animalis* subsp. *lactis* DSMZ 10140, and *B. animalis* subsp. *lactis* BB12) were grown in MRS broth at 37°C for 16 h in an anaerobic chamber (as described in 3.1.1). An aliquot (0.1 ml) of cultured medium was added to 10 ml dextrose-free MRS broth (described in section 3.1.1), and then centrifuged (15,000 g for 1 min). The supernatant was discarded. The bacterial cells were re-suspended in 1 ml of dextrose-free MRS broth, and inoculated (0.1 v/v) in a no-carbohydrate added MRS broth (no-CHO-added control), or in specially prepared MRS broths with 0.5% dextrose (positive control), ERS<sub>L-AMY</sub> or ERS<sub>VL-AMY</sub> from HAMS as the main carbohydrate source.

Dextrose, ERS<sub>L-AMY</sub> and ERS<sub>VL-AMY</sub> were added subsequent to autoclaving and cooling dextrose-free MRS to 50°C. To minimize microbial contamination, dextrose was added to the medium by filter sterilization, while ERS<sub>L-AMY</sub> and ERS<sub>VL-AMY</sub> were washed with 80% ethanol and exposed to UV light for 30 min before addition to the medium.

The inoculated media were incubated in anaerobic conditions, at 37°C for 72 h. At 0, 12, 24, 48 and 72 h, the inoculated media were carefully vortexed, and an aliquot (100 μl) was serially diluted in sterile peptone water (3M, St. Paul, MN) and spread-plated on MRS agar. The plates were incubated at 37°C under anaerobic conditions for 72 h, before counting the colony forming units (CFU).

All strains were grown on the different media in triplicate. The mean of two CFU values was considered one replicate. Within each experiment, each strain was grown each ERS, on the positive control or on the no-CHO-added control in triplicate starting on the same day.
3.10.2 Growth on ERS<sub>VL-AMY</sub> from four different starch materials

Three *Bifidobacterium* strains (*B. pseudolongum* ATCC 25526, *B. choerinum* ATCC 27686, and *B. infantis* ATCC 15697) were grown in MRS broth at 37°C for 16 h in an anaerobic chamber (as described in 3.1.1). The bacterial cells were re-suspended in 1 ml of dextrose-free MRS broth (as described in the previous section), and inoculated (0.1 v/v) in a dextrose-free MRS broth (no-CHO-added control), or in specially prepared MRS broths with 0.5% dextrose (positive control), ERS<sub>VL-AMY</sub> from HAMS, ERS<sub>VL-AMY</sub> from HMT, ERS<sub>VL-AMY</sub> from CCS 2%R-20U, or ERS<sub>VL-AMY</sub> from HAMS 2%R-20U as the main carbohydrate source.

Dextrose and the ERS<sub>VL-AMY</sub> were added to the dextrose-free MRS as described in the previous section. The inoculated media were incubated in anaerobic conditions, at 37°C for 72 h. CFU counts were obtained at 0, 12, 24, 48 and 72 h.

All strains were grown on the different media in triplicate. The mean of two CFU values was considered one replicate. Within each experiment, each strain was grown each ERS, on the positive control or on the no-CHO-added control in triplicate starting on the same day.
Chapter 4 : Results

4.1 Characterization of RS-containing preparations

4.1.1 General characteristics of the RS-containing preparations

When water was added to disperse the ethanol-precipitated CCS (prepared as in section 3.2.1), the dispersion immediately appeared transparent. The turbidity of the dispersion did not visually change over time, neither at 1 nor at 2% concentration. When the ethanol-precipitated CCS was rehydrated in the presence of variable levels of isoamylase, by the end of the 16 hour holding period the dispersion started to appear slightly opalescent and a white precipitate was observed.

When water was added to disperse the ethanol-precipitated HAMS, the dispersion immediately became turbid, with an almost immediate formation of a white precipitate. The rate of formation of the white precipitate tended to increase when the ethanol-precipitated HAMS was rehydrated in the presence of isoamylase.

After drying without rehydration, the ethanol precipitates formed a white soft pellet, easy to grind with a mortar and pestle. The samples obtained by rehydrating the ethanol precipitates were translucent and very hard to grind after drying, either in the presence or in the absence of isoamylase.

4.1.2 Starch recovery and resistant starch content

Starch recovery is shown in Table 4.1. For all the starch materials obtained by rehydrating the ethanol-precipitated CCS (NO R), starch yield was less than 50%. Recovery of dispersed ethanol-precipitated CCS was greater at 1% than at 2% concentration at rehydration, and for the samples rehydrated in presence of isoamylase. For the HAMS samples, starch recovery exceeded 77%, even at 1% concentration.
Table 4.1 Starch recovery of the resistant starch-containing preparations.
Common corn starch (CCS) and high-amylose maize starch (HAMS) were molecularly dispersed in 0.5N NaOH and precipitated with ethanol. The precipitate was either dried (NO R), or rehydrated at 1% (1%R) or 2% (2%R) starch concentration, in the presence of variable levels of isoamylase (10U or 20U).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CCS</td>
</tr>
<tr>
<td>NO R</td>
<td>96.1± 0.3</td>
</tr>
<tr>
<td>1%R</td>
<td>8.9± 2.7</td>
</tr>
<tr>
<td>1%R-10U</td>
<td>23.5± 3.0</td>
</tr>
<tr>
<td>1%R-20U</td>
<td>33.3± 2.7</td>
</tr>
<tr>
<td>2%R</td>
<td>33.8±12.6</td>
</tr>
<tr>
<td>2%R-10U</td>
<td>46.1± 4.8</td>
</tr>
<tr>
<td>2%R-20U</td>
<td>47.9± 4.8</td>
</tr>
</tbody>
</table>

Values are mean ± standard variation based on three replicates of the preparations.
Resistant starch (RS) content is presented in Table 4.2. For CCS, essentially no RS was observed for the ethanol precipitate (NO R), even after it was rehydrated (1%R and 2%R). Rehydration with 20U isoamylase led to about twice the levels of RS as compared to 10U isoamylase. For HAMS, some RS was generated by rehydrating the ethanol-precipitate in the absence of isoamylase (1%R or 2%R). In the presence of isoamylase, rehydration at 1% led to greater RS content as compared to rehydration at 2%.

Figure 4.1 shows the RS content of the RS-containing materials as a percentage of the precipitate and as a percentage of the starting material. For CCS, the 2%R-20U sample gave the highest RS starch yield (48%, Table 4.1) and the highest proportion of RS (18% of the precipitate was RS, which corresponds to approximately 10% of the starting material). For all the starch materials obtained by rehydrating the ethanol-precipitated HAMS in the presence of isoamylase, the proportion of RS was ~20% of the starting material.
Table 4.2 Resistant starch (RS) of the resistant starch-containing preparations. Common corn starch (CCS) and high-amylose maize starch (HAMS) were molecularly dispersed in 0.5N NaOH and precipitated with ethanol. The precipitate was either dried (NO R), or rehydrated at 1% (1%R) or 2% (2%R) starch concentration, in the presence of variable levels of isoamylase (10U or 20U).

<table>
<thead>
<tr>
<th>Sample</th>
<th>RS (%)</th>
<th>CCS</th>
<th>HAMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native starch</td>
<td>3.4±0.9</td>
<td>44.6±2.5</td>
<td></td>
</tr>
<tr>
<td>NO R</td>
<td>0.1±0.1</td>
<td>4.4±0.7</td>
<td></td>
</tr>
<tr>
<td>1%R</td>
<td>0.8±0.1</td>
<td>20.2±1.2</td>
<td></td>
</tr>
<tr>
<td>1%R-10U</td>
<td>9.5±1.4</td>
<td>25.6±1.7</td>
<td></td>
</tr>
<tr>
<td>1%R-20U</td>
<td>17.7±3.0</td>
<td>26.0±3.2</td>
<td></td>
</tr>
<tr>
<td>2%R</td>
<td>0.6±0.1</td>
<td>14.1±0.2</td>
<td></td>
</tr>
<tr>
<td>2%R-10U</td>
<td>9.4±2.0</td>
<td>19.0±1.3</td>
<td></td>
</tr>
<tr>
<td>2%R-20U</td>
<td>18.6±0.4</td>
<td>19.0±1.7</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± standard variation based on three replicates of the preparations.
Figure 4.1 Resistant starch (RS) content of the RS-containing materials.

Black bars represent the RS as a percentage of the precipitate. White bars represent the RS as a percentage of the starting material, obtained by multiplying the RS content of the precipitate by the decimal fraction of the recovery (Table 4.1). Common corn starch (CCS) and high-amylose maize starch (HAMS) were molecularly dispersed in 0.5N NaOH and precipitated with ethanol. The precipitate was either dried (NO R), or rehydrated at 1% (1%R) or 2% (2%R) starch concentration, in the presence of variable levels of isoamylase (10U or 20U).
4.1.3 Chemical characterization of the RS-containing starch preparations

4.1.3.1 Molecular size distribution

By size exclusion chromatography, for native starch granules the proportion of molecules eluting at the void volume region (k’<0.2) is generally considered to be amylopectin. The remaining proportion of molecules is generally considered amylase (Klucinec and Thompson, 1998). The iodine binding (λ\text{max}) serves as an indication of the chain length of the linear portion of the molecules eluting at a given k’’. The shorter the length of the linear portion, the lower the λ\text{max}.

Results of size exclusion chromatography for CCS and HAMS samples are shown in Fig. 4.2 and 4.3, respectively. For CCS, a lower proportion of amylopectin than in the original CCS was observed when the ethanol-precipitated CCS was rehydrated in the absence of isoamylase (1%R and 2%R). For HAMS, the proportion of amylopectin was somewhat smaller for the 2%R sample.

For both CCS and HAMS, no amylopectin peak was observed when the ethanol precipitates were rehydrated in the presence of isoamylase (10U and 20U), regardless of the concentration at rehydration. For CCS, the proportion of shorter linear chains was smaller for the samples rehydrated at 1%R (Fig. 4.2). The greatest proportion of shorter linear chains was detected for the CCS 2%R-20U sample.

For HAMS rehydrated at 1%R, the proportion of shorter linear chains was greater in the presence of isoamylase (Fig. 4.3). The greatest proportion of shorter linear chains was detected for the HAMS 1%R-20U sample.
Figure 4.2 Size exclusion chromatography of materials prepared from common corn starch (CCS).
Closed diamonds represent glucose concentration, and open circles represent the iodine binding. The dotted lines represent the molecular size distributions after multiplying the glucose concentration by the decimal fraction of the recovery (Table 4.1). CCS was molecularly dispersed in 0.5N NaOH and precipitated with ethanol. The precipitate was rehydrated at 1% (1%R) or 2% (2%R) starch concentration, in the presence of variable levels of isoamylase (10U or 20U).
Figure 4.3 Size exclusion chromatography of materials prepared from high-amylose maize starch (HAMS). Closed diamonds represent glucose concentration, and open circles represent the iodine binding. The dotted lines represent the molecular size distributions after multiplying the glucose concentration by the decimal fraction of the recovery (Table 4.1). HAMS was molecularly dispersed in 0.5N NaOH and precipitated with ethanol. The precipitate was rehydrated at 1% (1%R) or 2% (2%R) starch concentration, in the presence of variable levels of isoamylase (10U or 20U).
4.1.3.2 Chain length profiles of fully debranched molecules

For purposes of comparison, high-performance SEC chromatograms of the RS-containing materials (Fig. 4.4, 4.5, 4.6 and 4.7) were divided into four regions based on the retention times of the two response minima and the inflection for debranched CCS. For CCS, regions I, II, III and IV corresponded to 25.9, 20.4, 24.3 and 29.4% of the total area (Table 4.3). A very similar proportion of the chromatographic regions was observed for the two samples not treated with isoamylase during rehydration (1%R and 2%R).

The proportion of starch in region I for the CCS samples treated with isoamylase during rehydration (1%R-10U, 1%R-20U, 2%R-10U and 2%R-20U) was about double or more the one for CCS (Fig. 4.4, Table 4.3). However, when the chain length profile distributions were adjusted for the percentage of starting material (Fig. 4.5), the amount of starch in region I for those samples was less than in region I for CCS, and it decreased in the order: 1%R-20U ~ 2%R-20U > 2%R-10U > 1%R-10U (Table 4.3).

A lower proportion of starch in region III and IV was observed for the CCS samples treated with isoamylase during rehydration at 1% starch concentration (1%R-10U and 1%R-20U) than at 2% starch concentration (2%R-10U and 2%R-20U) (Table 4.2). Even for these last samples, the proportion of starch in region III and IV was only about 37 and 20%, respectively, of the corresponding regions for CCS.

For HAMS, regions I, II, III and IV corresponded to 53.8, 27.6, 12.3 and 6.3% of the total area (Table 4.4). The chromatograms of all the RS-containing materials prepared from HAMS all illustrate a higher starch peak in region I as compared to HAMS (Fig. 4.6 and 4.7). A slightly lower starch proportion in region III and IV was observed for the HAMS samples treated with isoamylase during rehydration at 1% starch concentration (1%R-10U and 1%R-20U) (Table 4.4).
Figure 4.4 Normalized chain length profiles of fully debranched RS-containing preparations from common corn starch (CCS). CCS was molecularly dispersed in 0.5N NaOH and precipitated with ethanol. The precipitate was rehydrated at 1% (1%R) or 2% (2%R) starch concentration, in the presence of variable levels of isoamylase (10U or 20U).
Figure 4.5 Recovery-adjusted chain length profiles of fully debranched RS-containing preparations from common corn starch (CCS), obtained by multiplying the normalized profile (Fig. 4.4) by the decimal fraction of the recovery (Table 4.1).

CCS was molecularly dispersed in 0.5N NaOH and precipitated with ethanol. The precipitate was rehydrated at 1% (1%R) or 2% (2%R) starch concentration, in the presence of variable levels of isoamylase (10U or 20U).
Figure 4.6 Normalized chain length profile of fully debranched RS-containing preparations from high-amylose maize starch (HAMS). HAMS was molecularly dispersed in 0.5N NaOH and precipitated with ethanol. The precipitate was rehydrated at 1% (1%R) or 2% (2%R) starch concentration, in the presence of variable levels of isoamylase (10U or 20U).
Figure 4.7 Recovery-adjusted chain length profiles of fully debranched RS-containing preparations from high-amylose maize starch (HAMS), obtained by multiplying the normalized profile (Fig. 4.6) by the decimal fraction of the recovery (Table 4.1).

HAMS was molecularly dispersed in 0.5N NaOH and precipitated with ethanol. The precipitate was rehydrated at 1% (1%R) or 2% (2%R) starch concentration, in the presence of variable levels of isoamylase (10U or 20U).
Table 4.3 Starch proportion in the chromatographic regions of fully debranched resistant starch-containing materials from common corn starch (CCS).

CCS was molecularly dispersed in 0.5N NaOH and precipitated with ethanol. The precipitate was rehydrated at 1\% (1\%R) or 2\% (2\%R) starch concentration, in the presence of variable levels of isoamylase (10U or 20U).

<table>
<thead>
<tr>
<th>CCS sample</th>
<th>Chromatographic regions</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
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<tr>
<td>Granular</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Wt %a</td>
<td>25.9</td>
<td>20.4</td>
<td>24.3</td>
<td>29.4</td>
<td></td>
</tr>
<tr>
<td>Recovery %b</td>
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<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>1%R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wt %</td>
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<td>24.7</td>
<td>30.2</td>
<td></td>
</tr>
<tr>
<td>Recovery %</td>
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<td>9.2</td>
<td>9.1</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>1%R-10U</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wt %</td>
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<td>18.9</td>
<td>14.0</td>
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</tr>
<tr>
<td>Recovery %</td>
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<td>21.8</td>
<td>13.6</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>1%R-20U</td>
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<tr>
<td>Wt %</td>
<td>66.7</td>
<td>15.2</td>
<td>10.9</td>
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<tr>
<td>Recovery %</td>
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<tr>
<td>2%R</td>
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<tr>
<td>Wt %</td>
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<td>20.1</td>
<td>24.2</td>
<td>29.1</td>
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<tr>
<td>Recovery %</td>
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<td>33.6</td>
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<tr>
<td>2%R-10U</td>
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</tr>
<tr>
<td>Wt %</td>
<td>44.5</td>
<td>22.7</td>
<td>19.1</td>
<td>13.7</td>
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<tr>
<td>Recovery %</td>
<td>79.1</td>
<td>51.3</td>
<td>36.3</td>
<td>21.5</td>
<td></td>
</tr>
<tr>
<td>2%R-20U</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wt %</td>
<td>46.5</td>
<td>23.0</td>
<td>19.2</td>
<td>11.3</td>
<td></td>
</tr>
<tr>
<td>Recovery %</td>
<td>85.9</td>
<td>53.9</td>
<td>37.9</td>
<td>18.5</td>
<td></td>
</tr>
</tbody>
</table>

\(a\) proportion (weight \%) of the chromatographic region. The basis for the percentage is the weight of the precipitated starch.

\(b\) proportion (weight \%) of the chromatographic region calculated from Fig. 4.5. The basis for the percentage is the original CCS. For example, in Fig. 4.5 the starch in region I may be compared for CCS and CCS 1\%R-10U. The proportion is 53.0\%.
Table 4.4 Starch proportion in the chromatographic regions of fully debranched resistant starch-containing materials from high-amylose maize starch (HAMS).

HAMS was molecularly dispersed in 0.5N NaOH and precipitated with ethanol. The precipitate was rehydrated at 1% (1%R) or 2% (2%R) starch concentration, in the presence of variable levels of isoamylase (10U or 20U).

<table>
<thead>
<tr>
<th>HAMS sample</th>
<th>Chromatographic regions</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granular</td>
<td></td>
<td>53.8</td>
<td>27.6</td>
<td>12.3</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>Wt %&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>Recovery %&lt;sup&gt;b&lt;/sup&gt;</td>
<td>82.8</td>
<td>80.5</td>
<td>82.0</td>
<td>77.0</td>
</tr>
<tr>
<td>1%R</td>
<td>Wt %</td>
<td>54.5</td>
<td>27.2</td>
<td>12.4</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>Recovery %</td>
<td>82.8</td>
<td>80.5</td>
<td>82.0</td>
<td>77.0</td>
</tr>
<tr>
<td>1%R-10U</td>
<td>Wt %</td>
<td>58.7</td>
<td>26.9</td>
<td>9.8</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>Recovery %</td>
<td>84.6</td>
<td>75.3</td>
<td>61.7</td>
<td>56.3</td>
</tr>
<tr>
<td>1%R-20U</td>
<td>Wt %</td>
<td>59.0</td>
<td>27.5</td>
<td>9.9</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>Recovery %</td>
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<td>83.1</td>
<td>67.0</td>
<td>47.1</td>
</tr>
<tr>
<td>2%R</td>
<td>Wt %</td>
<td>52.4</td>
<td>27.6</td>
<td>12.4</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>Recovery %</td>
<td>83.8</td>
<td>85.8</td>
<td>86.3</td>
<td>103.7</td>
</tr>
<tr>
<td>2%R-10U</td>
<td>Wt %</td>
<td>56.7</td>
<td>26.7</td>
<td>11.0</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>Recovery %</td>
<td>95.8</td>
<td>87.8</td>
<td>81.3</td>
<td>80.2</td>
</tr>
<tr>
<td>2%R-20U</td>
<td>Wt %</td>
<td>56.8</td>
<td>27.2</td>
<td>10.9</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>Recovery %</td>
<td>93.1</td>
<td>86.7</td>
<td>78.1</td>
<td>71.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> proportion (weight %) of the chromatographic region. The basis for the percentage is the weight of the precipitated starch.

<sup>b</sup> proportion (weight %) of the chromatographic region calculated from Fig. 4.7. The basis for the percentage is the original HAMS. For example, in Fig. 4.7 the starch in region I may be compared for HAMS and HAMS 1%R-10U. The proportion is 84.6%.
4.1.4 Physical characterization of the RS-containing preparations

4.1.4.1 Wide angle X-ray diffraction

For both CCS and HAMS, the ethanol precipitate (NO R) and the precipitate rehydrated in the absence of isoamylase (2%R) showed an essentially amorphous diffraction pattern (Fig. 4.8 and Fig. 4.9).

For CCS, the precipitate rehydrated in the presence of isoamylase showed a weak B-type crystalline pattern (Fig. 4.8). Rehydration with 20U isoamylase led to about twice the percentage of crystallinity as rehydration with 10U isoamylase (Table 4.5).

For HAMS, the precipitate rehydrated in presence of isoamylase (2%R-20U) showed an exceptionally weak B-type crystalline pattern (Fig. 4.9). For this sample, the peaks were weakly resolved, making it difficult to calculate proportion of crystallinity, which was estimated to be about 5% (Table 4.5).
Figure 4.8 Wide angle X-ray diffractograms for resistant starch-containing materials from common corn starch (CCS).

CCS was molecularly dispersed in 0.5N NaOH and precipitated with ethanol (NO R). The precipitate was rehydrated at 2% (2%R) starch concentration, in the presence of variable levels of isoamylase (10U or 20U). It is not clear what accounts for the peaks at angle greater than 30 ° for the NO R sample.
Figure 4.9 Wide angle X-ray diffractograms (WXRD) for resistant starch-containing materials from high-amylose maize starch (HAMS).

HAMS was molecularly dispersed in 0.5N NaOH and precipitated with ethanol (NO R). The precipitate was rehydrated at 2% (2%R) starch concentration, in the presence of isoamylase (20U). It is not clear what accounts for the peaks at angle greater than 30° for the NO R sample.
Table 4.5 Crystallinity calculated from wide angle X ray diffraction data for resistant starch-containing preparations.

Common corn (CCS) or high-amylose maize starch (HAMS) starch were molecularly dispersed in 0.5N NaOH and precipitated with ethanol. The precipitate was rehydrated at 2% (2%R) starch concentration, in the presence of variable levels of isoamylase (10U or 20U).

<table>
<thead>
<tr>
<th>Starch sample</th>
<th>% crystallinity</th>
<th>Mean&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCS NO R</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CCS 2%R</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CCS 2%R-10U</td>
<td>6.0</td>
<td>6.2± 0.2</td>
</tr>
<tr>
<td>CCS 2%R-20U</td>
<td>12.9</td>
<td>12.6± 0.2</td>
</tr>
<tr>
<td>HAMS NO R</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HAMS 2%R</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HAMS 2%R-20U</td>
<td>4.9</td>
<td>5.4± 0.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Two diffractograms were obtained for each sample, and the results were not distinguishable. Values are mean ± standard variation based on three different calculations for one diffractogram.
4.1.4.2 Solid-state $^{13}$C CP / MAS NMR

The results of the solid-state $^{13}$C CP/NMR analysis are shown in Fig. 4.10, 4.11 and 4.12. The carbons of the glucose monomers of starch have been assigned to specific positions in the NMR spectra with $C_1$ = 95-103 ppm; $C_2$, $C_3$, $C_5$ = a broad peak in the region 67-75 ppm; $C_4$ = 82 ppm; and $C_6$ = 63 ppm (Bociek and Gidley, 1985). Peak fitting software was used to fit 4-5 peaks, and these fitted peaks are also shown.

The peak position of fitted peaks is shown in Table 4.6. Data analysis for selected fitted peaks is shown in Table 4.7. For all the ethanol-precipitated samples (NO R), the $C_1$ peak was narrower, the $C_4$ peak was sharper, and the $C_6$ peak was less sharp than for the corresponding granular starches. When the ethanol-precipitated CCS and HAMS were rehydrated in the absence of isoamylase, the $C_4$ peak became slightly less sharp (Fig. 4.12 and 4.13).

For CCS, the $C_1$ peak of the sample rehydrated in the presence of isoamylase (CCS 2%R-20U) was broader (Fig. 4.12) and shifted towards lower ppm values (Table 4.6) as compared to the sample rehydrated in the absence of isoamylase (CCS 2%R). For HAMS, the fitted peak at 97 ppm of the sample rehydrated in presence of isoamylase (HAMS 2%R-20U) was less pronounced (Fig. 4.13) as compared to the sample rehydrated in the absence of isoamylase (HAMS 2%R) (Table 4.7).

The NMR spectra show a peak maximum for the $C_1$ peak at 101.9 ppm for the CCS 2%R-20U sample and at 103.5 ppm for the CCS 2%R sample (Table 4.7). A similar shift in the peak maximum for the $C_1$ peak was not observed for the HAMS preparations.
Figure 4.10 Solid-state $^{13}$C CP/NMR of granular waxy corn starch (WCS) and ethanol-precipitated waxy corn starch (WCS NO R).

Black lines are the original data. Green lines indicate the peaks fitted using Origin Software (version 8.1; Originlab, Massachusetts, USA), and red lines indicate the overall fit of the peak fitting operation. Four peaks were fitted at the positions of the peak maxima (ppm values are shown in Table 4.6) and one peak was fitted at 97 ppm. For WCS, no peak at 97 ppm could be successfully fit, so only the remaining four peaks were used. The WCS NOR was dispersed in 0.5N NaOH and precipitated with ethanol and then dried.
Figure 4.11 Solid-state $^{13}$C CP/NMR of granular common corn starch (CCS) and resistant starch-containing materials from CCS.

Black lines are the original data. Green lines indicate the peaks fitted using Origin Software (version 8.1; Originlab, Massachusetts, USA), and red lines indicate the overall fit of the peak fitting operation. Four peaks were fitted at the positions of the peak maxima (ppm values are shown in Table 4.6) and one peak was fitted at 97 ppm. CCS was molecularly dispersed in 0.5N NaOH and precipitated with ethanol (NO R). The precipitate was rehydrated at 2% (2%R) starch concentration, in the presence of isoamylase (20U). Both were then dried.
Figure 4.12 Solid-state $^{13}$C CP/NMR of granular high-amylose maize starch (HAMS) and resistant starch-containing materials from HAMS.

Black lines are the original data. Green lines indicate the peaks fitted using Origin Software (version 8.1; Originlab, Massachusetts, USA), and red lines indicate the overall fit of the peak fitting operation. Four peaks were fitted at the positions of the peak maxima (ppm values are shown in Table 4.6) and one peak was fitted at 97 ppm. HAMS was molecularly dispersed in 0.5N NaOH and precipitated with ethanol (NO R). The precipitate was rehydrated at 2% (2%R) starch concentration, in the presence of isoamylase (20U). Both were then dried.
Table 4.6 Peak maxima for the solid-state $^{13}$C CP/NMR spectra of granular starches and resistant starch-containing materials from waxy corn (WCS), common corn (CCS), or high-amylose maize starch (HAMS).

Non-granular starches were molecularly dispersed in 0.5N NaOH and precipitated with ethanol. The precipitate was rehydrated 2% (2%R) starch concentration, in the presence of isoamylase (20U).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chemical shifts$^a$</th>
<th>C1</th>
<th>C4</th>
<th>C2,3,5$^b$</th>
<th>C6</th>
</tr>
</thead>
<tbody>
<tr>
<td>WCS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granular</td>
<td></td>
<td>101.6</td>
<td>82.3</td>
<td>73.2</td>
<td>62.6</td>
</tr>
<tr>
<td>NO R</td>
<td></td>
<td>103.1</td>
<td>81.7</td>
<td>73.2</td>
<td>63.3</td>
</tr>
<tr>
<td>CCS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granular</td>
<td></td>
<td>102.3</td>
<td>82.1</td>
<td>73.2</td>
<td>62.7</td>
</tr>
<tr>
<td>NO R</td>
<td></td>
<td>103.7</td>
<td>82.8</td>
<td>73.2</td>
<td>62.4</td>
</tr>
<tr>
<td>2% R</td>
<td></td>
<td>103.5</td>
<td>82.5</td>
<td>73.2</td>
<td>62.2</td>
</tr>
<tr>
<td>2% R-20U</td>
<td></td>
<td>101.9</td>
<td>82.0</td>
<td>73.2</td>
<td>62.2</td>
</tr>
<tr>
<td>HAMS</td>
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<td></td>
</tr>
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<td>102.1</td>
<td>81.6</td>
<td>73.2</td>
<td>62.5</td>
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<tr>
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<td></td>
<td>103.5</td>
<td>82.4</td>
<td>73.2</td>
<td>61.5</td>
</tr>
<tr>
<td>2% R</td>
<td></td>
<td>103.2</td>
<td>82.3</td>
<td>73.2</td>
<td>62.8</td>
</tr>
<tr>
<td>2% R-20U</td>
<td></td>
<td>103.4</td>
<td>82.3</td>
<td>73.2</td>
<td>62.5</td>
</tr>
</tbody>
</table>

$^a$ values are peak maxima for the original data, before peak fitting analysis

$^b$ all spectra were adjusted slightly in order to align this peak due to minor variations in the magnetic field. The value of 73.2 was obtained from granular WCS analyzed immediately after analysis of the reference standard, and it was used to correct the ppm values for this C2,3,5 peak for the samples reported above.
Table 4.7 Peak parameters for fitted peaks for the solid-state $^{13}$C CP/NMR spectra of granular starches or resistant starch-containing materials from waxy corn (WCS), common corn (CCS), or high-amylose maize starch (HAMS).

Non-granular starches were molecularly dispersed in 0.5N NaOH and precipitated with ethanol. The precipitate was rehydrated at 1% (1%R) or 2% (2%R) starch concentration, in the presence of variable levels of isoamylase (10U or 20U).

<table>
<thead>
<tr>
<th>Samples</th>
<th>C1 $^b$</th>
<th>C4 $^b$</th>
<th>C6</th>
<th>Peak at 97ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>WCS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granular</td>
<td>WHH$^a$</td>
<td>6.8</td>
<td>8.2</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>PPA$^a$</td>
<td>15.1</td>
<td>11.1</td>
<td>8.9</td>
</tr>
<tr>
<td>NO R</td>
<td>WHH</td>
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<td>PPA</td>
<td>12.2</td>
<td>16.7</td>
<td>14.3</td>
</tr>
<tr>
<td>CCS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>WHH</td>
<td>6.3</td>
<td>7.2</td>
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<td>12.4</td>
<td>9.8</td>
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<td>WHH</td>
<td>5.4</td>
<td>6.9</td>
<td>11.0</td>
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<tr>
<td></td>
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<td>WHH</td>
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<td>8.2</td>
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<td></td>
<td>PPA</td>
<td>12.5</td>
<td>16.5</td>
<td>10.3</td>
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<tr>
<td>2%R-20U</td>
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<td>6.1</td>
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<tr>
<td></td>
<td>PPA</td>
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<td>7.9</td>
</tr>
<tr>
<td>HAMS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granular</td>
<td>WHH</td>
<td>6.9</td>
<td>7.7</td>
<td>6.7</td>
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<td>10.1</td>
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<tr>
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<td>PPA</td>
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<td>21.2</td>
<td>9.9</td>
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<tr>
<td>2%R</td>
<td>WHH</td>
<td>5.3</td>
<td>8.6</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>PPA</td>
<td>11.3</td>
<td>15.7</td>
<td>9.7</td>
</tr>
<tr>
<td>2%R-20U</td>
<td>WHH</td>
<td>5.8</td>
<td>7.7</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>PPA</td>
<td>13.3</td>
<td>17.0</td>
<td>7.2</td>
</tr>
</tbody>
</table>

$^a$ peak width at half height (WHH) and partial peak area (PPA) for fitted peaks

$^b$ coefficients of variation measured for WHH and PPA of each fitted peak for granular WCS (n=6) were between 3.1 and 9.5%.
4.1.4.3 Differential scanning calorimetry

DSC thermograms for CCS are shown in Fig. 4.13 and for HAMS are shown in Fig. 4.14. Data analysis is presented in Table 4.8. For both CCS and HAMS, the ethanol precipitate (NO R) exhibited a broad exotherm above 100°C during initial heating (H1) (Fig. 4.13 and 4.14), with a peak at about 140°C (Table 4.8). For these samples, the exothermic peak did not return completely to the baseline by 180°C, the final temperature reached during the thermal analysis, making it difficult to define the baseline. At a temperature above 180°C, starch molecular degradation can occur; therefore, the samples were not analyzed at temperature above 180°C. Due to the difficulties in defining the baseline for the exothermic peak, the calculated enthalpy value had a high variability. No peaks were observed during cooling (C) or reheating (H2) for these two samples (Fig. 4.13 and 4.14).

For both CCS and HAMS, the precipitate rehydrated in the absence of isoamylase (2%R) showed an exceptionally broad endotherm during initial heating (Fig. 4.13 and 4.14), with an enthalpy of 11.0 for CCS and 7.2 J/g for HAMS (Table 4.8).

For CCS, the precipitate rehydrated in the presence of isoamylase (2%R-20U) exhibited a less broad endotherm than for CCS 2%R. The CCS 2%R-20U sample had a lower peak temperature but higher enthalpy value during heating and reheating (Table 4.8). For CCS 2%R, no peaks were observed during cooling; while two exothermic peaks were observed for CCS 2%R-20U.

For HAMS, the precipitate rehydrated in the presence of isoamylase (2%R-20U) also exhibited a less broad endotherm than for HAMS 2%R (Table 4.8). The 2%R-20U had a higher peak temperature and higher enthalpy value during heating and reheating. For both HAMS 2%R and HAMS 2%R-20U, the enthalpy value during reheating was higher than during heating. During cooling, two exothermic peaks were observed for both HAMS 2%R and HAMS 2%R-20U.
Figure 4.13 Differential scanning calorimetry (DSC) thermograms for resistant starch-containing materials from common corn starch (CCS).

Initial heating (H1) was from 5 to 180°C, cooling (C) was from 180 to 5°C, and reheating (H2) was from 5 to 180°C. Each tick mark on the y-axis corresponds to 5 mW. Dashed lines represent the baselines used in the calculations. CCS was molecularly dispersed in 0.5N NaOH and precipitated with ethanol. The precipitate was either dried (NO R), or rehydrated at 2% (2%R) starch concentration, in the presence of variable levels of isoamylase (20U).
Figure 4.14 Differential scanning calorimetry (DSC) thermograms for resistant starch-containing materials from high-amylose maize starch (HAMS).

Initial heating (H1) was from 5 to 180°C, cooling (C) was from 180 to 5°C, and reheating (H2) was from 5 to 180°C. Each tick mark on the y-axis corresponds to 5 mW. Dashed lines represent the baselines used in the calculations. HAMS was molecularly dispersed in 0.5N NaOH and precipitated with ethanol. The precipitate was either dried (NO R), or rehydrated at 2% (2%R) starch concentration, in the presence of variable levels of isoamylase (20U).
Table 4.8 Thermal analysis of resistant starch-containing materials.

Common corn starch (CCS) or high-amylose maize starch (HAMS) were molecularly dispersed in 0.5N NaOH and precipitated with ethanol. The precipitate was either dried (NO R) or rehydrated at 2% (w/v) in the presence of variable levels of isoamylase (0 or 20 U/g starch).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial heating (H1)</th>
<th>Cooling (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak temperature (°C)</td>
<td>ΔH (J/g)</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>Mean</td>
</tr>
<tr>
<td>CCS</td>
<td>NO R</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2%R</td>
<td></td>
</tr>
<tr>
<td></td>
<td>92.4</td>
<td>99.4</td>
</tr>
<tr>
<td></td>
<td>2%R-20U</td>
<td></td>
</tr>
<tr>
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<td>100.7</td>
<td>101.1</td>
</tr>
<tr>
<td>HAMS</td>
<td>NO R</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2%R</td>
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</tr>
<tr>
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<td>100.7</td>
<td>101.1</td>
</tr>
<tr>
<td></td>
<td>2%R-20U</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100.7</td>
<td>101.1</td>
</tr>
</tbody>
</table>

*a no peak detected
Table 4.8 continued

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<tr>
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</table>

*a* no peak detected
4.2 Chemical characterization of the ERS obtained from the RS-containing preparations

4.2.1 Chain length profiles of fully debranched ERS

The recovered putative RS will hereafter be referred to as enzyme-resistant starch (ERS) to distinguish it from RS as an outcome of the RS determination method. Chain length profiles of debranched ERS obtained from the RS-containing materials prepared from CCS are shown in Fig. 4.15. The chain length profiles of the RS-containing preparations and the corresponding ERS from CCS are compared in Fig. 4.16. Chain length profiles of debranched ERS obtained from the RS-containing materials prepared from HAMS are shown in Fig. 4.17. The chain length profiles of the RS-containing preparations and the corresponding ERS from HAMS are compared in Fig. 4.18. For purposes of comparison, all high-performance SEC chromatograms were divided into four regions based on the retention times of the two response minima and the inflection point for debranched CCS (a previous figure, Fig. 4.4). Quantitative analyses of the data are provided in Table 4.9 and 4.10.

For all ERS obtained from the CCS and HAMS preparations, about 90% of the chain length profile was observed in regions II and III. The proportion of RS in region I was < 3.5% of the total area (Fig. 4.16 and 4.20, Table 4.9 and 4.10).

For the CCS preparations, ERS from the 1%R-10U and 1%R-20U samples had a very similar chain length distribution profile, with a peak at ~25 dp (Fig. 4.15). A different profile was observed for the samples obtained by rehydrating the ethanol-precipitated CCS at 2% starch concentration. Although ERS from 2%R-10U and 2%R-20U had chain length profiles in reasonable agreement with each other, with a peak at ~18 dp, the proportion of ERS in region I and II was lower for CCS 2%R-20U as compared to CCS 2%R-10U (Fig. 4.16, Table 4.9).
Figure 4.15 Chain length profiles of debranched ERS obtained from common corn starch (CCS) preparations.
These RS-containing preparations were made accordingly to the protocol of section 3.2.1. The original RS-containing preparations were rehydrated at either 1% or 2% solids, and with isoamylase levels of 10U or 20U/g of starch. Putative RS (ERS) was obtained by ethanol precipitation of the 16-hour digestate that resulted from the protocol of section 3.3.4.1.
Figure 4.16 Chain length profiles of fully debranched RS-containing preparations (filled lines) and corresponding RS (dotted lines) from common corn starch (CCS).
The RS profiles were obtained by multiplying the normalized profile (Fig. 4.15) by the decimal fraction of the RS (Table 4.2). The original RS-containing preparations were rehydrated at either 1% or 2% solids, and with isoamylase levels of 10U or 20U/g of starch. Putative RS was obtained by ethanol precipitation of the 16-hour digestate that resulted from the protocol of section 3.3.4.1.
Figure 4.17 Chain length profiles of debranched ERS obtained from high-amylose maize starch (HAMS) preparations. These RS-containing preparations were made accordingly to the protocol of section 3.2.1. The original RS-containing preparations were rehydrated at either 1% or 2% solids, and with isoamylase levels of 10U or 20U/g of starch. Putative RS (ERS) was obtained by ethanol precipitation of the 16-hour digestate that resulted from the protocol of section 3.3.4.1.
Figure 4.18 Chain length profiles of fully debranched RS-containing preparations (filled lines) and corresponding RS (dotted lines) from high-amylose maize starch (HAMS).

The RS profiles were obtained by multiplying the normalized profile (Fig. 4.19) by the decimal fraction of the RS (Table 4.2). The original RS-containing preparations were rehydrated at either 1% or 2% solids, and with isoamylase levels of 10U or 20U/g of starch. Putative RS was obtained by ethanol precipitation of the 16-hour digestate that resulted from the protocol of section 3.3.4.1.
For the HAMS preparations, ERS from the two samples not treated with isoamylase during rehydration (1%R and 2%R) had a very similar chain length distribution profile, with a peak at ~35 dp (Fig. 4.17). The profiles of the ERS from the samples treated with isoamylase during rehydration (1%R-10U, 1%R-20U, 2%R-10U, and 2%R-20U) were slightly different than the profiles of the ERS from the two samples not treated with isoamylase during rehydration. All those treated with isoamylase showed a very similar chain length distribution profile, with a peak at ~25 dp.

The chain length profile of debranched ERS obtained from HAMS is shown in Fig. 4.19. The chain length profiles of HAMS and of the ERS from HAMS are compared in Fig. 4.20. For the ERS obtained from native HAMS, the proportion of ERS in region I was much higher than for the ERS obtained from the CCS and HAMS preparations. The proportion of ERS in region I was about 17% of the initial native HAMS (Fig. 4.20, Table 4.10).
Figure 4.19 Chain length profile of debranched ERS obtained from high-amylose maize starch (HAMS).

Putative RS (ERS) was obtained by ethanol precipitation of the 16-hour digestate that resulted from the protocol of section 3.3.4.1.
Figure 4.20 Chain length profiles of fully debranched high-amylose maize starch (HAMS) (filled line) and corresponding RS (dotted line).

The RS profile was obtained by multiplying the normalized profile (Fig. 4.17) by the decimal fraction of the RS (Table 4.2). Putative RS was obtained by ethanol precipitation of the 16-hour digestate that resulted from the protocol of section 3.3.4.1.
Table 4.9 Starch proportion in the chromatographic regions of debranched ERS obtained from common corn starch (CCS) preparations.

These RS-containing preparations were made accordingly to the protocol of section 3.2.1. The original RS-containing preparations were rehydrated at either 1% or 2% solids, and with isoamylase levels of 10U or 20U/g of starch. Putative RS was obtained by ethanol precipitation of the 16-hour digestate that resulted from the protocol of section 3.3.4.1.

<table>
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<tr>
<th>CCS samples</th>
<th>Chromatographic regions</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1%R-10U</td>
<td>Wt %(^a)</td>
<td>3.0</td>
<td>46.7</td>
<td>41.6</td>
<td>8.7</td>
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<td></td>
<td>Recovery of RS %(^b)</td>
<td>0.3</td>
<td>4.4</td>
<td>3.9</td>
<td>0.8</td>
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<tr>
<td></td>
<td>Recovery %(^c)</td>
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<td>23.4</td>
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</tr>
<tr>
<td>1%R-20U</td>
<td>Wt %</td>
<td>3.0</td>
<td>46.7</td>
<td>41.6</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>Recovery of RS %</td>
<td>0.5</td>
<td>8.3</td>
<td>7.4</td>
<td>1.5</td>
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<tr>
<td></td>
<td>Recovery %</td>
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<td>2%R-10U</td>
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<tr>
<td></td>
<td>Recovery of RS %</td>
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<td>4.8</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Recovery %</td>
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<td>7.1</td>
</tr>
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<td>2%R-20U</td>
<td>Wt %</td>
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<td>13.9</td>
</tr>
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<td></td>
<td>Recovery of RS %</td>
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<td>10.2</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Recovery %</td>
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<td>24.4</td>
<td>53.3</td>
<td>22.8</td>
</tr>
</tbody>
</table>

\(^a\) proportion (weight %) of the chromatographic region. The basis for the percentage is the weight of the precipitated starch.

\(^b\) proportion (weight %) of the chromatographic region calculated by multiplying the decimal fraction of the RS in the RS-containing preparations (Table 4.2)

\(^c\) proportion (weight %) of the chromatographic region calculated from Fig. 4.16. The basis for the percentage is the original RS-containing starch preparation. For example, in Fig. 4.16 the starch in region I may be compared for the 1%R-10U sample and the RS from the 1%R-10U sample. The proportion is 0.5%. 
Table 4.10 Starch proportion in the chromatographic regions of debranched ERS obtained from high-amylose maize starch (HAMS) and HAMS preparations.

These RS-containing preparations were made accordingly to the protocol of section 3.2.1. The original RS-containing preparations were rehydrated at either 1% or 2% solids, and with isoamylase levels of 10U or 20U/g of starch. Putative RS (ERS) was obtained by ethanol precipitation of the 16-hour digestate that resulted from the protocol of section 3.3.4.1.

<table>
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<tr>
<th>Granular</th>
<th>Chromatographic regions</th>
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<th>IV</th>
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<td></td>
<td>HAMS samples</td>
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<td>Wt %</td>
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<td>44.0</td>
<td>28.0</td>
<td>7.4</td>
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<tr>
<td>Recovery %</td>
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<td>38.0</td>
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<tr>
<td>Adj wt %</td>
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<td>RS adj %</td>
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<td>1%R-10U</td>
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<td>41.0</td>
<td>6.9</td>
</tr>
<tr>
<td>Adj wt %</td>
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<td>0.8</td>
<td>12.6</td>
<td>10.5</td>
<td>1.8</td>
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<tr>
<td>RS adj %</td>
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<td>1.3</td>
<td>46.8</td>
<td>106.9</td>
<td>38.4</td>
</tr>
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<td>1%R-20U</td>
<td>Wt %</td>
<td>1.9</td>
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<td>42.6</td>
<td>8.4</td>
</tr>
<tr>
<td>Adj wt %</td>
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<td>0.5</td>
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<td>11.1</td>
<td>2.2</td>
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<td>112.1</td>
<td>61.6</td>
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* a proportion (weight %) of the chromatographic region. The basis for the percentage is the weight of the precipitated starch.

* b proportion (weight %) of the chromatographic region calculated by multiplying the decimal fraction of the RS in the RS-containing preparations (Table 4.2)

* c proportion (weight %) of the chromatographic region calculated from Fig. 4.18 for granular HAMS and from Fig. 4.20 for the RS-containing starch preparations. The basis for the percentage is the original RS-containing starch preparation. For example, in Fig. 4.20 the starch in region I may be compared for the 1%R-10U sample and the RS from the 1%R-10U sample. The proportion is 1.3%.
Table 4.10 (continued)

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<tr>
<td>Adj wt %</td>
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<td>12.9</td>
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<td>HAMS 2%R-10U</td>
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<tr>
<td>Wt %</td>
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<td>46.1</td>
<td>42.7</td>
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<tr>
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<td>RS adj %</td>
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<tr>
<td>Wt %</td>
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<td>42.7</td>
<td>8.9</td>
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<tr>
<td>Adj wt %</td>
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<td>8.1</td>
<td>1.7</td>
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<tr>
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<td>32.1</td>
<td>74.0</td>
<td>33.0</td>
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</tbody>
</table>

a proportion (weight %) of the chromatographic region. The basis for the percentage is the weight of the precipitated starch.

b proportion (weight %) of the chromatographic region calculated by multiplying the decimal fraction of the RS in the RS-containing preparations (Table 4.2)

c proportion (weight %) of the chromatographic region calculated from Fig. 4.20. The basis for the percentage is the original RS-containing starch preparation. For example, in Fig. 4.20 the starch in region I may be compared for the 2%R-10U sample and the RS from the 2%R-10U sample. The proportion is 0.2%.
4.3 Evaluation and characterization of RS prior to and after thermal treatment for selected RS-containing preparations with different particle sizes

4.3.1 RS content

The proportion of RS decreased from 35 to 17% for CCS 2%R-20U and from 42 to 23% for HAMS 2%R-20U, as particle size decreased (Fig. 4.21). The proportion of RS after thermal treatment was independent of particle size, about 14% for the CCS sample and 23% for the HAMS sample (Fig. 4.21).

A regression line for the proportion of RS as a function of particle size from the CCS and HAMS was obtained. The particle sizes used in the analysis were 125 μm or the average value of the range in Fig. 4.21. For CCS 2%R-20U, the y-intercept was calculated to be 13.6%; while, for HAMS 2%R-20U, the y-intercept was calculated to be 18.5%.
Figure 4.21 Resistant starch content as a function of particle size before (black bars) and after (grey bars) thermal treatment for CCS 2%R-20U (top figure) and HAMS 2%R-20U (bottom figure).

A regression line for the proportion of RS prior to thermal treatment as a function of particle size from the CCS and HAMS was obtained by using the particle of 125 μm or the average value of the range in the figure. For CCS 2%R-20U, the regression line equation was $y = 0.054x + 13.6$; while, for HAMS 2%R-20U, the regression line equation was $y = 0.052x + 18.5$. 
4.3.2 Chain length profiles of fully debranched ERS from RS-containing preparations of two particle sizes

For purposes of comparison, high-performance SEC chromatographs of the ERS and ERS after thermal treatment (TT-ERS) from HAMS and from the RS-containing materials were divided into four regions (Fig. 4.22, 4.23, and 4.24) based on the retention times of the two response minima and the flex point for debranched CCS (Fig. 4.4).

For all the RS-containing preparations, the overall shape of the ERS and TT-ERS chain length profile distribution was independent of particle size. All the TT-ERS chain length profiles had a higher proportion of starch in region I and II as compared to the ERS chain length profile for the respective non thermally treated ERS.

For the CCS preparations, both the ERS and the TT-ERS chain length distribution had a peak maximum at ~18 dp (Fig. 4.23). For the HAMS preparations, the ERS chain length distribution had a peak maximum at ~30 dp; while the TT-ERS chain length distribution had a peak at ~50 dp (Fig. 4.25).

For HAMS, the TT-ERS chain length distribution had a maximum peak at ~200 dp (Fig. 4.24). The ERS chain length profile distribution had a higher proportion of starch in region I and II as compared to the TT-ERS chain length profile distribution. Moreover, a portion of molecules in region I of ERS was of a somewhat longer dp (>200) as compared to TT-ERS.
Figure 4.22 Chain length profile distribution of fully debranched ERS from CCS 2% R-20U with two different particle sizes before and after thermal treatment. Samples were made accordingly to the protocol of section 3.2.2. The original RS-containing preparation was rehydrated at 2% solids and with isoamylase levels of 20U/g of starch. ERS was obtained by ethanol precipitation of the 16-hour digestate that resulted from the protocol of section 3.3.4.1. TT-ERS was obtained by thermally treating the RS-containing preparation at 100°C for 30 min prior to digestion (see section 3.3.2).
Figure 4.23 Chain length profile of debranched ERS from HAMS before and after thermal treatment.

ERS was obtained by ethanol precipitation of the 16-hour digestate that resulted from the protocol of section 3.3.4.1. TT-ERS was obtained by thermally treating the RS-containing preparation at 100°C for 30 min prior to digestion (see section 3.3.2).
Figure 4.24 Chain length profile distribution of fully debranched RS from HAMS 2%R-20U with two different particle sizes before and after thermal treatment. Samples were made accordingly to the protocol of section 3.2.2. The original RS-containing preparation was rehydrated at 2% solids and with isoamylase levels of 20U/g of starch. ERS was obtained by ethanol precipitation of the 16-hour digestate that resulted from the protocol of section 3.3.4.1. TT-ERS was obtained by thermally treating the RS-containing preparation at 100°C for 30 min prior to digestion (see section 3.3.2).
4.4 Determination of the time course of digestion for selected RS-containing starches

4.4.1 Fisher F-test for best fit analysis

The time course of digestion data for HAMS, HMT, CCS 2%R-20U, and HAMS 2%R-20U were fitted by using a double exponential decay model with 5 parameters (Eq. 1 in section 3.3.3.2). Although the double exponential decay model with 5 parameters gave a good fit (r> 0.978) for all the starches analyzed (Table 4.11), experimental values at times > 24 hours for CCS 2%R-20U and HAMS 2%R-20U were lower than predicted by the model (Fig. 1B in appendix B). For the HAMS and the HMT, the experimental values did not appear to systematically differ from the predicted values (Fig. 1B in appendix B).

To search for a better model, the time course of digestion data for all starches were also fitted by using a triple exponential decay model with 6 parameters (Eq. 2 in section 3.3.3.2). The quality of two fits can be compared by using the Fisher F-test (Neter et al. 1996). Results of the Fisher F-test for best fit comparison of the double exponential decay model with 5 parameters and the triple exponential decay model with 6 parameters are shown in Table 4.11. For HAMS and HMT, the p-value of the Fisher F-test for best fit obtained comparing the double exponential 5 parameter model vs. the triple exponential 6 parameter model was 1.0; while for CCS 2%R-20U, and HAMS 2%R-20U the p-value was <0.01. The Fisher F-test for best fit compares the fit of two equations, asking whether the more complex model (the one with more parameters) fits better (has a smaller sum-of-squares) than the less complex model (the one with less parameters) (Neter et al. 1996). If the less complex model is a better fit, the calculated F-value is <1.0. If the F-value is >1.0, there are two possibilities: a) the more complex model is a better fit; b) the less complex model is a better fit, but random scatter led the more complicated model to better fit the data. To distinguish between these two possibilities, the p-value is calculated. If the p-value is <0.05, the more complex model is considered to be a significantly better fit than the simpler model (Neter et al. 1996). For CCS 2%R-20U and HAMS 2%R-20U, statistical analysis indicated that the triple exponential decay
Table 4.11 Comparison of goodness of fit for different exponential models to describe the time course of digestions

<table>
<thead>
<tr>
<th>Starch</th>
<th>Model equation (parameters)</th>
<th>r</th>
<th>DF&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SS&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
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<td></td>
<td>Triple exponential (6)</td>
<td>0.9892</td>
<td>41</td>
<td>548.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HMT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Double exponential (5)</td>
<td>0.9909</td>
<td>42</td>
<td>311.9</td>
<td>0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.0&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Triple exponential (6)</td>
<td>0.9909</td>
<td>41</td>
<td>311.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HAMS 2%R-20U</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Double exponential (5)</td>
<td>0.9823</td>
<td>42</td>
<td>675.8</td>
<td>24.64&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&lt; 0.01&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Triple exponential (6)</td>
<td>0.9892</td>
<td>41</td>
<td>415.9</td>
<td>9.62 *10&lt;sup&gt;-6&lt;/sup&gt;f</td>
<td>1.0&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Triple exponential (7)</td>
<td>0.9892</td>
<td>40</td>
<td>415.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCS 2%R-20U</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Double exponential (5)</td>
<td>0.9728</td>
<td>42</td>
<td>1145.0</td>
<td>20.35&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&lt; 0.01&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Triple exponential (6)</td>
<td>0.9819</td>
<td>41</td>
<td>765.2</td>
<td>0.0778&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.0&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Triple exponential (7)</td>
<td>0.9820</td>
<td>40</td>
<td>763.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Degrees of freedom  
<sup>b</sup> Sum of squares of the residuals  
<sup>c</sup> F value calculated as  
\[
F-value = \frac{(SS_1 - SS_2)/SS_2}{(DF_1 - DF_2)/DF_2}
\]  
where the subscript 1 refers to the model with less parameters, and the subscript 2 refers to the model with more parameters  
<sup>d</sup> p-value calculated by using “The Free Statistics Calculators Website” (<i>Online Software</i>, http://www.danielsoper.com/statcalc/)  
<sup>e</sup> F value and p-value obtained comparing the double exponential (5 parameter) model vs. the triple exponential (6 parameter) model  
<sup>f</sup> F value and p-value obtained comparing the triple exponential (6 parameter) model vs. the triple exponential (7 parameter) model
model with 6 parameters gave a significant better fit than the double exponential decay equation with 5 parameters. For HAMS and HMT, the more complex model was not an improvement.

For CCS 2%R-20U and HAMS 2%R-20U, a triple exponential decay model with 7 parameters (Eq. 3 in section 3.3.3.2) was selected to fit the time course of digestion data (Table 4.11). The Fisher F-test was used to compare the fitting of the triple exponential decay model with 6 parameters and the triple exponential decay model with 7 parameters. Statistical analysis indicated that the triple exponential decay model with 7 parameters did not give a significantly better fit than the triple exponential decay equation with 6 parameters.

**4.4.2 Kinetics of digestion**

The double exponential decay equation (with 5 parameters) was used to fit the time course of digestion data for HAMS and HMT (Fig. 4.25). The triple exponential decay equation (with 6 parameters) was used to fit the time course of digestion data for CCS 2%R-20U and HAMS 2%R-20U (Fig. 4.25). Visual comparison of the time course of digestion fits shows that CCS 2%R-20U and HAMS 2%R-20U are digested more rapidly than HAMS and HMT in the first ~3 hours of the digestion.

The values for the 6 or 7 parameters from the kinetic analysis of the time course of digestion for the four starches is shown in Table 4.12. A $S_{ND}$ value was estimated for HAMS and HMT. The $S_{ND}$ value for HMT was higher than for HAMS. For CCS 2%R-20U and HAMS 2%R-20U, the $k_{1a}$ was at least 60 fold greater than the $k_1$. The $k_1$ and $k_2$ for CCS 2%R-20U and HAMS 2%R-20U were of the same order of magnitude than the $k_1$ and $k_2$ for HAMS and HMT.
Figure 4.25 Time course of digestion of the extended resistant starch assay for HAMS, HMT, CCS 2%R-20U and HAMS 2%R-20U.

RS was determined from the aliquot of sample removed at 16 h of digestion. Curves for HAMS and HMT were obtained using a 5-parameter, double-exponential equation; curves for CCS 2%R-20U and HAMS 2%R-20U were obtained using a 6-parameter, triple-exponential equation. Curves shown are best fits of analysis of pooled data from 3 independent digestions. Parameters for these curves are shown in Table 4.12.
Table 4.12 Values for the 5 or 6 parameters from the kinetic analysis of the time course of digestion of the extended resistant starch assay.

$S_t$ is the percentage of undigested starch; $t$ is the time; $S_{Ia}$, $S_I$ and $S_2$ are the percentage of three distinct starch substrates; and $k_{Ia}$, $k_I$ and $k_2$ are the reaction rate constants for the decay of substrates $S_{Ia}$, $S_I$ and $S_2$. $S_{ND}$ corresponds to the percentage of residual starch, a fraction predicted to not be digested by pancreatic $\alpha$-amylase.

<table>
<thead>
<tr>
<th>Starch</th>
<th>$S_{Ia}$</th>
<th>$k_{Ia}$</th>
<th>$S_I$</th>
<th>$k_I$</th>
<th>$S_2$</th>
<th>$k_2$</th>
<th>$S_{ND}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAMS$^b$</td>
<td>29.3± 5.7</td>
<td>0.50± 0.15</td>
<td>49.9± 7.8</td>
<td>3.6± 1.7*10^{-2}</td>
<td>18.7± 9.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMT$^b$</td>
<td>27.9± 3.3</td>
<td>1.1± 0.2</td>
<td>24.4± 2.9</td>
<td>8.7± 2.5*10^{-2}</td>
<td>46.3± 1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCS 2%R-20U$^c$</td>
<td>35.2± 3.8</td>
<td>68.8± 18.4</td>
<td>31.3± 3.0</td>
<td>1.6± 0.4</td>
<td>33.6± 1.6</td>
<td>1.1± 0.3*10^{-2}</td>
<td>-</td>
</tr>
<tr>
<td>HAMS 2%R-20U$^c$</td>
<td>26.5± 2.7</td>
<td>95.5± 30.8</td>
<td>35.5± 2.1</td>
<td>1.5± 0.2</td>
<td>38.0± 1.1</td>
<td>7.3± 1.5*10^{-3}</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$Values are expressed as mean ± standard variation, n=3, for 3 independent digestions.

$^b$Parameters for HAMS and HMT were obtained using the double exponential equation $S_t = S_{Ia} e^{-k_{Ia}t} + S_I e^{-k_I t} + S_{ND}$

$^c$Parameters for CCS 2%R-20U and HAMS 2%R-20U were obtained using the triple exponential equation $S_t = S_{Ia} e^{-k_{Ia}t} + S_I e^{-k_It} + S_2 e^{-k_2t}$
4.5 Estimation of extracellular amylolytic activity of pure cultures of *Bifidobacterium* strains by an agar plate assay

4.5.1 Development of a soluble-starch agar plate assay

Two procedures from the literature (Ryan et al. 2006, Wang et al. 1999b) were combined and modified to develop a soluble-starch agar plate assay (see section 3.6.1.1). Aliquots of the grown cultures of *B. animalis* subsp. *lactis* Lafti B94, *B. animalis* subsp. *lactis* DSMZ 10140, and *B. pseudolongum* ATCC 25526 were either transferred to a sterile paper discs placed on the surface of the agar plate (according to Wang et al., 1999), or to an individual well formed in the agar plate (according to Ryan et al., 2006). Results of the assay are shown in Table 4.13.

For both inoculum techniques and at all inoculum levels tested, inoculation of *B. animalis* subsp. *lactis* Lafti B94 and *B. pseudolongum* ATCC 25526 on soluble starch-containing agar plates led to the formation of unstained regions around the disk or the well, after iodine staining (Fig. 4.26). These regions will be referred to as *unstained zones*.

No unstained zones were evident for *B. animalis* subsp. *lactis* DSMZ 10140, regardless of the inoculum technique or the inoculum level. For *B. animalis* subsp. *lactis* Lafti B94 and *B. pseudolongum* ATCC 25526, the radius of the unstained zones increased with increasing the volume of the inoculation (Table 4.13).
Table 4.13 Radius of unstained zones formed after 2 days for three *Bifidobacterium* strains incubated at 37°C in soluble starch-containing agar plates.

<table>
<thead>
<tr>
<th><em>Bifidobacterium</em> species</th>
<th>Inoculation technique</th>
<th>Aliquot inoculated (µl)</th>
<th>Radius of unstained zone (mm)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. animalis</em> subsp. <em>lactis</em> Lafti B94</td>
<td>Disk on agar plate</td>
<td>15</td>
<td>3.0± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>2.5± 0.7</td>
</tr>
<tr>
<td></td>
<td>Well on agar plate</td>
<td>15</td>
<td>3.5± 1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>2.9± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>1.0± 0.7</td>
</tr>
<tr>
<td><em>B. animalis</em> subsp. <em>lactis</em> DSMZ 10140</td>
<td>Disk on agar plate</td>
<td>15</td>
<td>No zone(^b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>No zone</td>
</tr>
<tr>
<td></td>
<td>Well on agar plate</td>
<td>15</td>
<td>No zone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>No zone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>No zone</td>
</tr>
<tr>
<td><em>B. pseudolongum</em> ATCC 25526</td>
<td>Disk on agar plate</td>
<td>15</td>
<td>12.5± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>10.5± 0.7</td>
</tr>
<tr>
<td></td>
<td>Well on agar plate</td>
<td>15</td>
<td>11.0± 1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>10.0± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>8.5± 3.5</td>
</tr>
</tbody>
</table>

\(^a\) For cultures inoculated on the disk, the radius of the zone was measured from the border of the disk. For cultures inoculated in the wells, the radius of the zone was measured from the border of the well. The shortest and largest radius for each disk or well was averaged. Values are expressed as mean ± standard variation for three independent incubations.

\(^b\) No unstained zone was visible
B. animalis subsp. lactis
DSMZ 10140

B. pseudolongum
ATCC 25526

B. animalis subsp. lactis
Lafti B94

Figure 4.26 Photograph of unstained zones as visualized by staining the soluble starch-containing agar plates with iodine solution following incubation with cultures of *B. animalis* subsp. *lactis* Lafti B94, *B. animalis* subsp. *lactis* DSMZ 10140, and *B. pseudolongum* ATCC 25526. For *B. pseudolongum* ATCC 25526, the unstained zone is indicated by the dotted line in the figure.
4.5.2 Development of a native HAMS and ERS agar plate assay

To test native HAMS or ERS as the added carbohydrate in the medium, it was necessary to add them after sterilizing the medium. To test whether microbial contamination was effectively eliminated by washing HAMS or ERS from HAMS (prepared as described in section 3.3.4.1) with 80% ethanol and exposing them to UV light for 30 min before addition to the sterilized medium, 10 plates using HAMS or ERS from HAMS were prepared and incubated in an anaerobic chamber at 37°C for 4 days. No microbial contamination was observed on any of the agar plates tested.

The native HAMS and the ERS from HAMS-containing agar plates were then inoculated with the grown cultures of *B. animalis* subsp. *lactis* Lafti B94, *B. animalis* subsp. *lactis* DSMZ 10140, and *B. pseudolongum* ATCC 25526 and either stained with an iodine solution or with iodine vapor. When HAMS-containing agar plates were stained with the iodine solution, no zones were visible for any of the strains tested. However, when HAMS-containing agar plates were stained with iodine vapor (Fig. 4.27), light regions around the wells were observed for *B. animalis* subsp. *lactis* Lafti B94 and *B. pseudolongum* ATCC 25526. These regions will be referred to as light zones. No distinct light zone was visible for *B. animalis* subsp. *lactis* DSMZ 10140 by either iodine staining methods. Results of the assay are shown in Table 4.14.

No zones were visible for any of the strains tested when ERS from HAMS-containing agar plates were stained either with the iodine solution or the iodine vapor.
Figure 4.27 Photograph of light zones as visualized by staining the HAMS-containing agar plates with iodine vapor following incubation with cultures of *B. animalis* subsp. *lactis* Lafti B94, *B. animalis* subsp. *lactis* DSMZ 10140, and *B. pseudolongum* ATCC 25526. The light zones are indicated by the dotted lines in the figure. Enhancement contrast has been used to enhance the unstained zones in the figure.
Table 4.14 Radius of light zones formed after 4 days incubation at 37°C for three *Bifidobacterium* strains in HAMS-containing agar plates.

Plates were stained by exposing the plates to iodine vapor generated from ~2 g of iodine crystals for 30 min at room temperature.

<table>
<thead>
<tr>
<th><em>Bifidobacterium</em> strain</th>
<th>Radius of light zone (mm)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. animalis</em> subsp. <em>lactis</em> Lafti B94</td>
<td>7.1± 1.2</td>
</tr>
<tr>
<td><em>B. animalis</em> subsp. <em>lactis</em> DSMZ 10140</td>
<td>No zone$^b$</td>
</tr>
<tr>
<td><em>B. pseudolongum</em> ATCC 25526</td>
<td>11.8± 1.2</td>
</tr>
</tbody>
</table>

$^a$ Radius of the zone measured from the border of the well. Values are expressed as mean ± standard variation for three independent incubations.

$^b$ After staining with iodine vapor, no light zone was visible
4.5.3 Use of agar plate assays to estimate the activity of a pancreatic $\alpha$-amylase solution

When only pancreatic $\alpha$-amylase was placed in soluble starch or granular HAMS-containing agar plate wells, the radius of the zones on both linearly increased with the log of the activity of the pancreatic $\alpha$-amylase added (Fig. 4.28). For the same level of enzyme activity, the light zone formed with granular HAMS was of greater radius than the unstained zone formed with soluble starch.

4.5.4 Use of agar plate assays to estimate the extracellular amylolytic activity of strains of Bifidobacterium

Of the 37 Bifidobacterium strains tested (see Table. 3.1), six did not form an unstained zone on the soluble starch-containing agar plate (Table 4.15). This result was interpreted as an inability of the tested strain to synthesize extracellular enzymes capable of degrading soluble starch.

The 31 strains that tested positive for degradation of soluble starch were also inoculated on a granular HAMS. The presence of a light zone following iodine staining was observed for all the 31 strains tested (Table 4.15). The radius of the zones ranged from 1.0 to 11.7 mm on soluble starch and from 5.5 to 16.3 mm on granular HAMS-containing agar plates. For each strain, the light zones formed on the granular HAMS-containing agar plates were always of greater radius than the unstained zones observed on the soluble starch-containing agar plates.

B. choerinum ATCC 27686 and B. pseudolongum ATCC 25526 formed the zones with the largest radius on soluble starch (radius of the zone 11.7 and 10.4 mm, respectively) and on granular HAMS-containing agar plates (radius of the zone 16.3 and 12.5 mm, respectively). Relative to other strains, B. infantis ATCC 15697 formed zones of intermediate radius (5.0 mm) on soluble starch; while it formed zones of a comparatively larger radius (13.1 mm) on granular HAMS.
Figure 4.28 Effect of added pancreatic α-amylase activity on the radius of the zones formed on agar plates containing either soluble starch (♦) or granular HAMS (○) following 4 days incubation.

Curves shown are best fits of analysis of pooled data from 3 independent analyses for each enzyme activity.
Table 4.15 Zone formation on agar plates containing soluble starch or granular HAMS for 37 *Bifidobacterium* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Radius of the zone (mm)</th>
<th>Soluble starch (unstained zone(^a))</th>
<th>Granular HAMS (light zone(^b))</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 15703</td>
<td><em>B. adolescentis</em></td>
<td>6.1±1.1</td>
<td>9.8±1.1</td>
<td></td>
</tr>
<tr>
<td>ATCC 25527</td>
<td><em>B. animalis</em></td>
<td>4.8±2.2</td>
<td>11.2±0.9</td>
<td></td>
</tr>
<tr>
<td>ATCC 27672</td>
<td><em>B. animalis</em></td>
<td>6.9±1.2</td>
<td>10.0±2.0</td>
<td></td>
</tr>
<tr>
<td>ATCC 27536</td>
<td><em>B. animalis subsp. lactis</em></td>
<td>2.9±1.9</td>
<td>8.3±0.5</td>
<td></td>
</tr>
<tr>
<td>Lafti B 94</td>
<td><em>B. animalis subsp. lactis</em></td>
<td>2.7±1.2</td>
<td>7.0±1.4</td>
<td></td>
</tr>
<tr>
<td>BB 12</td>
<td><em>B. animalis subsp. lactis</em></td>
<td>3.6±0.8</td>
<td>8.9±0.7</td>
<td></td>
</tr>
<tr>
<td>DSMZ 10140</td>
<td><em>B. animalis subsp. lactis</em></td>
<td>No zone(^c)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>RB 4825</td>
<td><em>B. animalis subsp. lactis</em></td>
<td>2.4±1.1</td>
<td>6.7±0.4</td>
<td></td>
</tr>
<tr>
<td>RB 5251</td>
<td><em>B. animalis subsp. lactis</em></td>
<td>1.0±0.9</td>
<td>5.5±0.6</td>
<td></td>
</tr>
<tr>
<td>RB 5733</td>
<td><em>B. animalis subsp. lactis</em></td>
<td>2.2±0.7</td>
<td>8.5±1.1</td>
<td></td>
</tr>
<tr>
<td>RB 7339</td>
<td><em>B. animalis subsp. lactis</em></td>
<td>3.0±1.1</td>
<td>7.9±0.4</td>
<td></td>
</tr>
<tr>
<td>ATCC 15696</td>
<td><em>B. bifidum</em></td>
<td>No zone(^c)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>ATCC 29521</td>
<td><em>B. bifidum</em></td>
<td>No zone(^c)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>RB 3046</td>
<td><em>B. bifidum</em></td>
<td>4.8±3.1</td>
<td>6.7±1.6</td>
<td></td>
</tr>
<tr>
<td>RB 5422</td>
<td><em>B. bifidum</em></td>
<td>2.5±1.5</td>
<td>6.1±1.5</td>
<td></td>
</tr>
<tr>
<td>ATCC 15698</td>
<td><em>B. breve</em></td>
<td>8.0±0.9</td>
<td>9.5±3.2</td>
<td></td>
</tr>
<tr>
<td>ATCC 15700</td>
<td><em>B. breve</em></td>
<td>4.3±1.4</td>
<td>7.5±0.7</td>
<td></td>
</tr>
<tr>
<td>RB 4753</td>
<td><em>B. breve</em></td>
<td>3.1±1.1</td>
<td>8.5±1.6</td>
<td></td>
</tr>
<tr>
<td>RB 5333</td>
<td><em>B. breve</em></td>
<td>6.7±1.0</td>
<td>11.4±1.1</td>
<td></td>
</tr>
<tr>
<td>RB 8613</td>
<td><em>B. breve</em></td>
<td>1.4±1.2</td>
<td>6.9±1.1</td>
<td></td>
</tr>
<tr>
<td>ATCC 27686</td>
<td><em>B. choerinum</em></td>
<td>11.7±0.9</td>
<td>16.3±2.1</td>
<td></td>
</tr>
<tr>
<td>RB 1791</td>
<td><em>B. choerinum</em></td>
<td>No zone(^c)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>RB 4052</td>
<td><em>B. choerinum</em></td>
<td>1.5±2.3</td>
<td>7.5±1.9</td>
<td></td>
</tr>
<tr>
<td>RB 4536</td>
<td><em>B. choerinum</em></td>
<td>3.4±1.1</td>
<td>8.9±1.3</td>
<td></td>
</tr>
<tr>
<td>ATCC 15697</td>
<td><em>B. infantis</em></td>
<td>5.0±2.0</td>
<td>13.1±2.9</td>
<td></td>
</tr>
<tr>
<td>ATCC 25962</td>
<td><em>B. infantis</em></td>
<td>No zone(^c)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>RB 0171</td>
<td><em>B. infantis</em></td>
<td>2.8±1.3</td>
<td>6.9±1.6</td>
<td></td>
</tr>
<tr>
<td>RB 1281</td>
<td><em>B. infantis</em></td>
<td>1.1±0.5</td>
<td>7.4±1.6</td>
<td></td>
</tr>
<tr>
<td>RB 7239</td>
<td><em>B. infantis</em></td>
<td>1.5±1.0</td>
<td>6.6±1.3</td>
<td></td>
</tr>
<tr>
<td>RB 9632</td>
<td><em>B. infantis</em></td>
<td>1.7±1.3</td>
<td>9.2±2.7</td>
<td></td>
</tr>
<tr>
<td>ATCC 15707</td>
<td><em>B. longum</em></td>
<td>1.7±1.7</td>
<td>7.1±1.8</td>
<td></td>
</tr>
<tr>
<td>ATCC 15708</td>
<td><em>B. longum</em></td>
<td>3.4±1.0</td>
<td>8.7±0.8</td>
<td></td>
</tr>
<tr>
<td>RB 1280</td>
<td><em>B. longum</em></td>
<td>1.2±0.7</td>
<td>7.9±0.9</td>
<td></td>
</tr>
<tr>
<td>RB 3982</td>
<td><em>B. longum</em></td>
<td>No zone(^c)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>RB 5859</td>
<td><em>B. longum</em></td>
<td>1.2±0.8</td>
<td>5.5±1.1</td>
<td></td>
</tr>
<tr>
<td>RB 9321</td>
<td><em>B. longum</em></td>
<td>4.7±1.4</td>
<td>9.3±1.6</td>
<td></td>
</tr>
<tr>
<td>ATCC 25526</td>
<td><em>B. pseudolongum</em></td>
<td>10.4±0.4</td>
<td>12.5±0.5</td>
<td></td>
</tr>
</tbody>
</table>

See footnotes on next page
Table 4.15 (continued)

a unstained region around the well
b light region around the well
c the six strains that failed to produce a unstained zone on soluble starch-containing agar plates were not tested with HAMS-containing agar plates
4.6 Estimation of soluble extracellular amylolytic enzyme activity for selected strains of *Bifidobacterium* grown in the presence of different carbohydrate substrates

Cell-free supernatant (CFS), obtained as described in section 3.7, from six strains of bifidobacterium was investigated in detail. No zones were observed following incubation of the CFS of *B. animalis* subsp. *lactis* DSMZ 10140 after being grown on glucose, soluble starch or granular HAMS (Table 4.16). For all other organisms, CFS produced at least small zones after incubation with each substrate. Relatively large unstained zones were observed following incubation of the CFS of *B. choerinum* ATCC 27686 and *B. pseudolongum* ATCC 25526 for all the growth substrates tested. For *B. pseudolongum* ATCC 25526, the radius of the unstained zones was greater when granular HAMS, as compared to glucose or soluble starch, was used as growth substrate (Table 4.16).

For all six strains investigated, unstained zones were observed following incubation of the CFS after being grown on ERS from HAMS. Moreover, a unstained zone was observed also for the uninoculated ERS from HAMS-containing broth (Table 4.16).

The radii of the unstained zones formed by the unfiltered culture and the CFS when grown in presence of glucose are compared in Table 4.17. For all six strains investigated, CFS produced zones of smaller radius than the unfiltered culture.
Table 4.16 Estimation of soluble amylolytic activity after inoculation of cell free supernatant (CFS) of five *Bifidobacterium* strains that had been grown on broth with one of four different carbohydrate sources: glucose, soluble starch, HAMS, or ERS from HAMS.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Bifidobacterium</th>
<th>Different growth substrates for generating CFS&lt;sup&gt;b&lt;/sup&gt;</th>
<th>glucose</th>
<th>soluble starch</th>
<th>HAMS</th>
<th>ERS from HAMS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Exp. 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Exp. 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Exp. 1</td>
<td>Exp. 2</td>
</tr>
<tr>
<td>BB12</td>
<td><em>B. animalis</em> subsp. <em>lactis</em></td>
<td>&lt;0.5, &lt;0.5</td>
<td>&lt;0.5, &lt;0.5</td>
<td>&lt;0.5, &lt;0.5</td>
<td>&lt;0.5, &lt;0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>DSMZ 10140</td>
<td><em>B. animalis</em> subsp. <em>lactis</em></td>
<td>No zone</td>
<td>No zone</td>
<td>No zone</td>
<td>No zone, No zone</td>
<td>2.7</td>
</tr>
<tr>
<td>ATCC 27686</td>
<td><em>B. choerinum</em></td>
<td>3.7</td>
<td>3.3</td>
<td>2.0, 2.3</td>
<td>2.4, 3.8</td>
<td>3.2</td>
</tr>
<tr>
<td>ATCC 15697</td>
<td><em>B. infantis</em></td>
<td>&lt;0.5</td>
<td>&lt;0.5, &lt;0.5</td>
<td>&lt;0.5, &lt;0.5</td>
<td>&lt;0.5, &lt;0.5</td>
<td>3.3</td>
</tr>
<tr>
<td>ATCC 25526</td>
<td><em>B. pseudolongum</em></td>
<td>0.8</td>
<td>1.8</td>
<td>0.8, 2.3</td>
<td>2.2, 3.1</td>
<td>2.9</td>
</tr>
<tr>
<td>Negative control&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>No zone</td>
<td>No zone</td>
<td>No zone, No zone</td>
<td>2.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> CFS was obtained by centrifuging the broth (after 24h incubation) and subsequently filtering the supernatant to remove any residual cells or insoluble starch. In experiment 1 (Exp. 1), CFS was directly transferred to an individual well in a prepared soluble starch-containing agar plate. In experiment 2 (Exp. 2), CFS was first concentrated (about two fold) and then transferred to an individual well in a prepared soluble starch-containing agar plate. Unstained zones were visualized by staining the soluble starch-containing agar plates with an iodine solution.

<sup>b</sup> values are the radius (mm) of the unstained zone on soluble starch. The two related experiments were performed once.

<sup>c</sup> the negative control was filtered broth that had not been inoculated.

<sup>d</sup> the value <0.5 indicates that a small zone was observed, but it was difficult to quantify.
Table 4.17 Estimation of soluble amylolytic activity after inoculation of the unfiltered culture or the cell free supernatant (CFS) of five *Bifidobacterium* strains that had been grown on glucose as main carbohydrate sources.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Bifidobacterium</th>
<th>Inoculation typea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>unfiltered culture</td>
</tr>
<tr>
<td>BB12</td>
<td><em>B. animalis</em> subsp. <em>lactis</em></td>
<td>3.4</td>
</tr>
<tr>
<td>DSMZ 10140</td>
<td><em>B. animalis</em> subsp. <em>lactis</em></td>
<td>No zone</td>
</tr>
<tr>
<td>ATCC 27686</td>
<td><em>B. choerinum</em></td>
<td>11.5</td>
</tr>
<tr>
<td>ATCC 15697</td>
<td><em>B. infantis</em></td>
<td>5.4</td>
</tr>
<tr>
<td>ATCC 25526</td>
<td><em>B. pseudolongum</em></td>
<td>10.1</td>
</tr>
</tbody>
</table>

a values are the radius (mm) of the unstained zone on soluble starch. Unstained zones were visualized by staining the soluble starch-containing agar plates with an iodine solution. The experiment was performed once.

b CFS was obtained by centrifuging the broth (after 24h incubation) and subsequently filtering the supernatant to remove any residual cells or insoluble starch.
4.7 Estimation of residual amylolytic activity in isolated RS

4.7.1 Extent of residual amylolytic activity released by ERS and ERS_{L-AMY}

The HAMS that resisted 16 hours digestion (defined as RS) was obtained either by ethanol precipitation (ERS), as described in the official method for *in vitro* RS determination (AOAC 2002.02, AACC 32-40, see section 3.3.4.1), or by centrifugation without ethanol addition (as described in section 3.3.4.2) (ERS_{L-AMY}). The proportion of ERS and ERS_{L-AMY} recovered from HAMS was not different (53.1± 3.1 and 51.3± 0.8, respectively).

The extent of amylolytic activity of recovered RS from HAMS was indirectly measured by the soluble-starch agar plate assay. ERS or ERS_{L-AMY} was added to the wells as described in section 3.8.1. The released activity was 10 fold higher for ERS than for ERS_{L-AMY} (Table 4.18).

The residual amylolytic activity was estimated for ERS_{L-AMY} from four RS-containing starch materials (Table 4.19). Of the four ERS_{L-AMY} tested, ERS_{L-AMY} from HAMS 2%R-20U had the lowest residual amylolytic activity (Table 4.19). For all the ERS_{L-AMY} tested, ~90% of the amylolytic activity estimated by utilizing the soluble-starch agar plate assay remained associated with the starch after the initial 1h period. Whether all the activity was released during the 4-day incubation of the clearing zone assay was not tested.
Table 4.18 Estimation of amylolytic activity released by ERS and ERS<sub>L-AMY</sub> from HAMS\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Resistant starch</th>
<th>Radius (mm) of the unstained zone</th>
<th>Estimated released amylolytic activity (U/ml)\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERS from HAMS</td>
<td>12.6± 0.5</td>
<td>1.41± 0.21</td>
</tr>
<tr>
<td>ERS&lt;sub&gt;L-AMY&lt;/sub&gt; from HAMS</td>
<td>8.5± 0.1</td>
<td>0.14± 0.01</td>
</tr>
</tbody>
</table>

\textsuperscript{a} ERS was obtained following HAMS digestion by ethanol precipitation as described in the official method for \textit{in vitro} RS determination (AOAC 2002.02, AACC 32-40). ERS<sub>L-AMY</sub> was obtained following HAMS digestion by centrifugation. ERS and ERS<sub>L-AMY</sub> were added to a MRS broth at 0.5% starch. The released amylolytic activity was estimated by using the soluble-starch agar plate assay.

\textsuperscript{b} amylolytic activity extrapolated by utilizing the curve in Fig. 4.28 as standard curve, interpreted as follow:

\[ \text{Radius of the zones} = 4.0503 \times \log(\alpha\text{-amylase activity (U/ml)}) + 11.957 \]
Table 4.19 Estimated amylolytic activity released by ERS<sub>L-AMY</sub> obtained four RS-containing starch materials<sup>a</sup>.

<table>
<thead>
<tr>
<th>Materials tested</th>
<th>ERS&lt;sub&gt;L-AMY&lt;/sub&gt;-containing broth</th>
<th>Starch-free supernatant</th>
<th>Calculated activity for ERS&lt;sub&gt;L-AMY&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity (U/ml)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Activity (U/ml)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(U/ml)&lt;sup&gt;c&lt;/sup&gt; (%)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>HAMS</td>
<td>0.19± 0.04</td>
<td>0.022± 0.018</td>
<td>0.17</td>
</tr>
<tr>
<td>HMT</td>
<td>0.21± 0.15</td>
<td>0.016± 0.012</td>
<td>0.20</td>
</tr>
<tr>
<td>CCS 2%R-20U</td>
<td>0.42± 0.12</td>
<td>0.026± 0.019</td>
<td>0.39</td>
</tr>
<tr>
<td>HAMS 2%R-20U</td>
<td>0.04± 0.01</td>
<td>0.003± 0.001</td>
<td>0.037</td>
</tr>
</tbody>
</table>

<sup>a</sup> ERS<sub>L-AMY</sub> was obtained following starch digestion by centrifugation. ERS<sub>L-AMY</sub> was added to a MRS broth at 0.5% starch. The released amylolytic activity was estimated by using the soluble-starch agar plate assay.

<sup>b</sup> Amylolytic activity extrapolated by utilizing the curve in Fig. 4.28 as standard curve, interpreted as follow:

\[ \text{Radius of the zones} = 4.0503 \times \log(\alpha\text{-amylase activity (U/ml)}) + 11.957 \]

<sup>c</sup> Calculated activity released over time during the agar plate assay, estimated as:

\[ \text{Activity of ERS}_{L-AMY} = \text{activity of ERS}_{L-AMY}\text{-containing broth} - \text{activity of starch-free supernatant} \]

<sup>d</sup> Apparent proportion not in the supernatant of the initial starch suspension but released over time during the agar plate assay, calculated as:

\[ \% \text{ activity released by ERS}_{L-AMY} \text{ overtime} = \left( \frac{\text{activity of ERS}_{L-AMY}}{\text{activity of ERS}_{L-AMY}\text{-containing broth}} \right) \times 100 \]
4.7.2 Further digestion of ERS\textsubscript{L-AMY}

Further digestion of the recovered ERS\textsubscript{L-AMY} was tested in two ways: 1) the ERS\textsubscript{L-AMY} was treated as the starch in the official method for in vitro RS determination (AOAC 2002.02, AACC 32-40, see section 3.3.4.1), after addition of pancreatic $\alpha$-amylase in maleate buffer, and 2) as in the official method for in vitro RS determination, except that no additional pancreatic $\alpha$-amylase was added to the maleate buffer. Visual comparison of the time course of digestion shows that for the ERS\textsubscript{L-AMY} from HAMS, from CCS 2\%R-20U and from HAMS 2\%R-20U digestion was more rapid in the enzyme-containing buffer than in the buffer without added enzyme (Fig. 4.29). Little difference was observed in the digestion of ERS\textsubscript{L-AMY} from HMT in the two buffers.

Assuming that the ERS\textsubscript{L-AMY} from the four RS-containing starch materials tested was constituted by a single substrate, the time course of digestion data for the enzyme-containing buffer and the buffer without added enzyme were fitted by using a single exponential decay model (Fig. 4.29). The single exponential decay model gave a reasonably good fit ($r^2 > 0.666$) for all the samples (Fig. 4.29). A more detailed statistical analysis was not performed, because of the relatively few time points collected during the time course of digestion.
ERS<sub>L-AMY</sub> from HAMS

ERS<sub>L-AMY</sub> from HMT

ERS<sub>L-AMY</sub> from CCS 2%R-20U

ERS<sub>L-AMY</sub> from HAMS 2%R-20U

Figure 4.29 Time course of further digestion of ERS<sub>L-AMY</sub> from HAMS, HMT, CCS 2%R-20U and HAMS 2%R-20U.

The ERS<sub>L-AMY</sub> was treated as the starch in the official method for in vitro RS determination (AOAC 2002.02, AACC 32-40, see section 3.3.4.1), after addition of pancreatic α-amylase in maleate buffer (●), or without additional pancreatic α-amylase added to the maleate buffer (○). Curves were obtained using a single exponential equation ($S_t = S_1 e^{-kt}$). Curves shown are best fits of analysis of pooled data from 2 independent digestions.
4.8 Reduction of amylolytic activity released from starch resistant to 16 h digestion

The effect of resuspending the ERSL-AMY from HAMS in potassium thiocyanate (0.4 M), EDTA (0.1%), or SDS (0.1%) solutions was evaluated in two ways by using the clearing zone assay (section 3.8.1). After the ERSL-AMY from HAMS was resuspended in the three solutions, it was collected by centrifugation and added to MRS, and 1) the starch-containing MRS broth was put into the wells, or 2) the starch-free MRS supernatant (SFS) was generated and put into the wells. Resuspension of ERSL-AMY from HAMS in either SDS or EDTA solutions led to a reduction of the released amylolytic activity estimated for the ERSL-AMY-containing broth and the SFS (Table 4.20). Resuspension in EDTA solution appeared to be the most effective treatment, with a more than 10-fold reduction of the released amylolytic activity as compared to the starch prior the treatment.

The effect of an extended EDTA treatment was also evaluated in the two ways previously described by using the clearing zone assay (section 3.8.1). The released amylolytic activity of the broth containing ERSL-AMY from HAMS was reduced by ~90% by incubating the samples with 0.1% EDTA at 37°C for 1 hour (Fig. 4.30). Extending the treatment time further reduced the amylolytic activity released by the starch-containing broth.

The ERSL-AMY from HAMS, HMT, CCS 2%R-20U and HAMS 2%R-20U were incubated in 0.1% EDTA at 37°C for 24 hours to produce ERSVL-AMY. For each ERSVL-AMY, the EDTA treatment reduced the released amylolytic activity by more than 95% in the starch-containing broth (Table 4.21).
Table 4.20 Estimated amylolytic activity released by ERS₁₋₅MY from HAMS following resuspension in potassium thiocyanate, EDTA or SDS solutions\(^a\).

<table>
<thead>
<tr>
<th>Resuspension treatment</th>
<th>Material tested</th>
<th>Activity (U/ml)(^b)</th>
<th>Activity (U/ml)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Starch-containing broth</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Activity (U/ml)(^b)</td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td></td>
<td>0.19± 0.04</td>
<td>0.022± 0.018</td>
</tr>
<tr>
<td>Potassium thiocyanate (0.4 M)</td>
<td></td>
<td>0.21± 0.03</td>
<td>0.030± 0.019</td>
</tr>
<tr>
<td>EDTA (0.1%)</td>
<td></td>
<td>0.013± 0.009</td>
<td>0.002± 0.001</td>
</tr>
<tr>
<td>SDS (0.1%)</td>
<td></td>
<td>0.040± 0.019</td>
<td>0.004± 0.001</td>
</tr>
<tr>
<td></td>
<td>Starch-free supernatant</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Activity (U/ml)(^b)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) ERS₁₋₅MY was obtained following HAMS digestion by centrifugation. Following resuspension, the treated starch was added to a MRS broth at 0.5% starch. After 1 hour, the starch-free supernatant was prepared by filtering an aliquot of the starch-containing broth. The released amylolytic activity was estimated by using the soluble-starch agar plate assay.

\(^b\) released amylolytic activity extrapolated by utilizing the curve in Fig. 4.28 as standard curve, interpreted as follow:

\[ \text{Radius of the zones} = 4.0503 \times \log(\alpha\text{-amylose activity (U/ml)}) + 11.957 \]
Figure 4.30 Residual amylolytic activity released by ERS_L-AMY from HAMS as a function of hours of incubation in the EDTA solution.

ERS_L-AMY was obtained following HAMS digestion by centrifugation. Following resuspension, the treated starch was added to a MRS broth at 0.5% starch. After 1 hour, the starch-free supernatant was prepared by filtering an aliquot of the starch-containing broth. The released amylolytic activity was estimated by using the soluble-starch agar plate assay.
Table 4.21 Estimated amylolytic activity released by ERS\textsubscript{VL-AMY} and ERS\textsubscript{L-AMY} from HAMS, HMT, CCS2%R-20U and HAMS 2%R-20U\textsuperscript{a}.

<table>
<thead>
<tr>
<th>ERS\textsubscript{L-AMY} from Materials tested</th>
<th>Calculated released activity reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERS\textsubscript{VL-AMY}-containing broth</td>
<td>ERS\textsubscript{L-AMY}-containing broth</td>
</tr>
<tr>
<td>Activity (U/ml)\textsuperscript{b}</td>
<td>Activity (U/ml)\textsuperscript{c}</td>
</tr>
<tr>
<td>HAMS</td>
<td>0.0016±0.0001</td>
</tr>
<tr>
<td>HMT</td>
<td>0.0019±0.0004</td>
</tr>
<tr>
<td>CCS 2%R-20U</td>
<td>0.0028±0.0003</td>
</tr>
<tr>
<td>HAMS 2%R-20U</td>
<td>0.0017±0.0007</td>
</tr>
</tbody>
</table>

\textsuperscript{a} ERS\textsubscript{L-AMY} was obtained following HAMS digestion by centrifugation. ERS\textsubscript{VL-AMY} was obtained by incubating ERS\textsubscript{L-AMY} in EDTA solution for 24 h. ERS\textsubscript{VL-AMY} or ERS\textsubscript{L-AMY} were added to a MRS broth at 0.5% starch. The released amylolytic activity was estimated by using the soluble-starch agar plate assay.

\textsuperscript{b} released amylolytic activity extrapolated by utilizing the curve in Fig. 4.28 as standard curve, interpreted as follow:

\[ \text{Radius of the zones} = 4.0503 \times \log(\text{\textgreek{a}-amylase activity (U/ml)}) + 11.957 \]

\textsuperscript{c} as reported in table 4.19

\textsuperscript{d} reduction of released amylolytic activity following EDTA treatment, calculated as:

\[ \text{Reduction (\%)} = \left( \frac{\text{activity of ERS\textsubscript{L-AMY}-containing broth} - \text{activity of ERS\textsubscript{VL-AMY}-containing broth}}{\text{activity of ERS\textsubscript{L-AMY}-containing broth}} \right) \times 100 \]
4.9 Effect of residual amylolytic activity of \( \text{ERS}_{L-}\text{AMY} \) from HAMS on the growth of selected strains of \textit{Bifidobacterium} when ERS is the main carbohydrate source

Three strains of \textit{Bifidobacterium} (\textit{B. pseudolongum} ATCC 25526, \textit{B. animalis} subsp. \textit{lactis} DSMZ 10140, \textit{B. animalis} subsp. \textit{lactis} BB12 were selected for their different ability to degrade starch, as shown in Table 4.15.

Of the three strains of \textit{Bifidobacterium} investigated, \textit{B. pseudolongum} ATCC 25526 had the greatest ability to degrade starch. For \textit{B. pseudolongum}, all carbohydrate sources analyzed allowed growth by 24 h relative to a no-carbohydrate-added MRS (No-CHO-added) (Fig. 4.31). By 48 h, highest counts were observed for the dextrose (positive control), and the lowest for the negative control. By 72 h, the viable cell counts for \( \text{ERS}_{L-}\text{AMY} \) and \( \text{ERS}_{VL-}\text{AMY} \) were similar to the No-CHO-added control, while counts remained higher for the positive control. For the entire incubation time, there was no difference in viable cell counts for \( \text{ERS}_{L-}\text{AMY} \) as compared to \( \text{ERS}_{VL-}\text{AMY} \).

For \textit{B. animalis} subsp. \textit{lactis} DSMZ 10140, the carbohydrate sources analyzed allowed growth by 12 h, not distinguishable from the No-CHO-added control. By 24 h, the viable cell counts for \( \text{ERS}_{L-}\text{AMY} \) and \( \text{ERS}_{VL-}\text{AMY} \) were somewhat higher than for the No-CHO-added control, but much lower than for the positive control.

For \textit{B. animalis} subsp. \textit{lactis} BB12, all carbohydrate sources analyzed allowed slight growth through 12 h, not distinguishable from the No-CHO-added control. By 24 h, the viable cell counts for \( \text{ERS}_{L-}\text{AMY} \) were higher than \( \text{ERS}_{VL-}\text{AMY} \) and No-CHO-added control, and not distinguishable from the positive control. On the contrary, at incubation time \( \geq 24 \) h, the viable cell counts for \( \text{ERS}_{VL-}\text{AMY} \) were higher than for \( \text{ERS}_{L-}\text{AMY} \).

For both \textit{B. animalis} subsp. \textit{lactis} DSMZ 10140 and \textit{B. animalis} subsp. \textit{lactis} BB12, the viable cell counts at incubation time \( \geq 24 \) h decreased more rapidly for the no-CHO-added control than for either \( \text{ERS}_{L-}\text{AMY} \) or \( \text{ERS}_{VL-}\text{AMY} \).
Figure 4.31 Effect of residual amylolytic activity on ERS from HAMS on viable cells counts of *B. pseudolongum* ATCC 25526, *B. animalis* subsp. *lactis* DSMZ 10140 and *B. animalis* subsp. *lactis* BB12.

The no-carbohydrate-added control (No-CHO-added control, ■) was dextrose-free MRS broth. Test MRS broths contained 0.5% carbohydrate. The main carbohydrate source were either dextrose (positive control, ♦), ERS<sub>L-AMY</sub> from HAMS (△) or ERS<sub>VL-AMY</sub> from HAMS (X). All strains were grown on the four media in triplicate, and points represent mean, with error bars representing the standard variation.
4.10 Growth of selected strains of *Bifidobacterium* on ERS$_{VL-AMY}$ from four different sources

*B. pseudolongum* ATCC 25526, *B. infantis* ATCC 15697, and *B. choerinum* ATCC 27686 were selected as the three strains of *Bifidobacterium* with the greatest ability to degrade HAMS, as shown in Table 4.15.

For *B. pseudolongum*, by 24 h all carbohydrate sources analyzed allowed more growth than the no-carbohydrate-added MRS (no-CHO-added control) (Fig. 4.32). By 72 h, counts for ERS$_{VL-AMY}$ from CCS 2%R-20U and from HAMS 2%R-20U were both still higher than for the no-CHO-added control.

For *B. choerinum*, by 12 h all carbohydrate sources analyzed allowed more growth than the no-CHO-added control. By 24 h, the viable cell counts for ERS$_{VL-AMY}$ from HMT were similar to the no-CHO-added control, while counts remained higher for ERS$_{VL-AMY}$ from HAMS, CCS 2%R-20U, and HAMS 2%R-20U.

For *B. infantis*, growth on each type of ERS$_{VL-AMY}$ analyzed was similar to the no-CHO-added control through 48 h.

For all strains, counts in viable cells at 72 h were lower for the ERS$_{VL-AMY}$ from HMT than for the other ERS$_{VL-AMY}$. 


Figure 4.32 Effect of ERS obtained from different RS-containing starch materials on viable cells counts of *B. pseudolongum* ATCC 25526, *B. choerinum* ATCC 27686, *B. infantis* ATCC 15697.

The no-carbohydrate-added control (No-CHO-added control, ■) was dextrose-free MRS broth. Test MRS broths contained 0.5% carbohydrate. The main carbohydrate source was either 0.5% dextrose (positive control, ♦), ERS<sub>VL-AMY</sub> from HAMS (X), from HMT (▲), from HAMS 2%R-20U (○), or from CCS 2%R-20U (●). All strains were grown on the six media in triplicate, and points represent mean, with error bars representing the standard variation.
Chapter 5 : Discussion

5.1 Influence of preparation conditions on the chemical structure of the RS-containing starch preparations

5.1.1 Effect of rehydration of the ethanol precipitates on the starch molecules recovery and molecular distribution

RS-containing starch materials were prepared by rehydration of an ethanol precipitate of common corn starch (CCS) or high-amylose maize starch (HAMS) that had been molecularly dispersed in 0.5N NaOH. The ethanol precipitation of the dispersed starch resulted in a recovery greater than 95% (Table 4.1). Evans and Thompson (2008) described how ethanol may serve to cause near-complete precipitation of starch.

For the rehydration treatment, water was added to disperse the ethanol precipitates. For ethanol-precipitated CCS, the addition of water did not cause immediate turbidity, but for the HAMS samples the dispersion immediately became turbid. Turbidity is related to formation of aggregates with a size similar to the wavelength of light (Gidley and Bulpin 1989). Thus, rehydration of the ethanol precipitates led to the formation of at least a few large aggregates for the HAMS samples, but not for the CCS samples.

Starch molecular aggregation in aqueous systems is enhanced by increasing the amylose content over the amyllopectin content of the dispersion (Adkins and Greenwood 1966, Klucinec and Thompson 1999, Zobel 1988). The CCS used in this study is about 27% amylose, while the HAMS is about 73% amylose (Rees, 2008). The greater molecular aggregation observed upon rehydration of the ethanol-precipitated HAMS could be attributable to higher amylose content of HAMS as compared to CCS. However, other factors, perhaps the chain length distribution and molecular size of amyllopectin and the molecular size of amylose, could be important as well.
Starch molecules may disperse but they can not be said to dissolve in water. Once the ethanol precipitates are rehydrated to re-disperse the starch, starch-starch interactions are preferred to the starch-water interactions, and thus the starch molecules in water tend to aggregate and precipitate. Double helices and aggregated double helices are associated with this retrogradation behavior (Gidley and Bulpin 1989, Klucinec and Thompson 1999). Double helices may aggregate and organize into crystallites (Miles et al. 1984, Ring et al. 1987). Intermolecular double helices and/or crystallites formed from the double helices of more than one molecule are referred to as physical junction zones (Klucinec and Thompson 2002). Figure 5.1 shows how amylose and amylopectin can associate and aggregate in water. In the current study, regardless of the starch concentration (1% or 2%) at rehydration, precipitation and recovery of starch molecules from dispersion was greater for the HAMS than for the CCS samples (Table 4.1). This outcome shows that dispersed HAMS has a greater tendency toward molecular aggregation than dispersed CCS.

Starch aggregation in aqueous systems is known to be enhanced by increasing concentration in the range 0.2-5.0% (Gidley and Bulpin 1989). Gidley and Bulpin (1989) showed that for synthetic amylose, the aggregation of amylose in water may result in precipitation or gelation, depending on the chain length and concentration of the amylose. Gelation was favored by longer chain lengths and higher concentrations. In the current study, the recovered starch was in form of a dense precipitate, suggesting that precipitation was the predominant phase, regardless of the starch concentration (1% or 2%) at rehydration. Nonetheless, recovery of starch after rehydration of the ethanol precipitates was greater at 2%R than at 1%R (Table 4.1), particularly for dispersed ethanol-precipitated CCS (Fig. 4.1). By doubling the starch concentration at rehydration, the likelihood that two starch molecules will form a physical junction zone increases. Physical junction zones are commonly understood to be the basis of molecular aggregation that leads to gelation or precipitation from dispersion. Thus, increasing the starch concentration at rehydration leads to greater starch recovery.
Figure 5.1 Association and aggregation of starch molecules in water. For purpose of simplicity, only the terminal portion of an amylopectin molecule is shown.
For the CCS 1%R and 2%R samples, a greater proportion of starch molecules eluting after the void volume region (defined as $k' > 0.2$) was observed by size exclusion chromatography (SEC) as compared to CCS (Fig. 4.2). By SEC, molecules eluting after the void volume region are generally considered to be amylose (Klucinec and Thompson 1998). However, chromatograms of fully debranched CCS (whether 1%R or 2%R) samples by high performance size exclusion chromatography (HPSEC) were very similar to the chromatograms of fully debranched CCS (Fig. 4.2). Klucinec and Thompson (1998) showed that for CCS, starch molecules eluting in region I of the HPSEC chromatograms are mainly amylose. Because the proportion of starch eluting in region I of the CCS 1%R and 2%R samples was very similar to the proportion of starch eluting in region I of CCS (Table 4.3), no evidence for an increased proportion of amylose in the CCS 1%R and 2%R samples was observed by HPSEC. The smaller region I peak by HPSEC as expected from the SEC chromatogram would suggest that some of the starch molecules eluting after the void volume region by SEC were amylopectin molecules of smaller size.

For both CCS and HAMS, the HPSEC profiles of 1%R and 2%R samples were similar to the profiles of the corresponding starting material (Fig. 4.5 and 4.6). Therefore, for both CCS and HAMS, amylose and amylopectin tended to coprecipitate from dispersion in a proportion similar to the amylose/amylopectin proportion of the starting material.

The behavior of amylose and amylopectin mixtures in aqueous dispersion is still unclear. Some studies of gelled mixtures of amylose and amylopectin suggest the interaction between amylose and amylopectin, leading to mixed amylose-amylopectin physical junction zones (Adkins and Greenwood 1966, Gudmundsson and Eliasson 1990, Klucinec and Thompson 1999, Klucinec and Thompson 2002, Rindlav-Westling et al. 2002). Phase separation of amylose and amylopectin at 80°C has been documented by Kalichevsky and Ring (1987). Other studies (Biliaderis 1998, Doublier and Llamas 1993, Leloup et al. 1991) supported the incompatibility of the two starch molecules in an aqueous medium. Since amylopectin in dilute aqueous dispersions is far more stable to retrogradation than amylose (Adkins and Greenwood 1966, Zobel 1988), a preferential precipitation of amylose would be expected as a consequence of amylose and
amylopectin phase separation. The coprecipitation of amylose and amylopectin in proportion similar to the proportion in the starting material seems inconsistent with a phase separation.

5.1.2 Effect of rehydration in the presence of isoamylase on starch molecules recovery and molecular distribution

RS-containing starch materials were also prepared by rehydrating the ethanol precipitate in the presence of variable levels of isoamylase. Isoamylase catalyzes debranching, the cleavage of $\alpha(1\rightarrow6)$-linkages. Debranching of amylopectin generates short linear molecules with average chain lengths of ~20 dp for CCS and ~32 dp for HAMS (Takeda et al. 1993, Yun and Matheson 1993). Debranching of amylose generates linear molecules with average chain lengths of ~300 dp for CCS and ~220 dp for HAMS (Klucinec and Thompson 1998, Takeda et al. 1989).

For CCS, greater starch recovery was observed when the ethanol precipitate was rehydrated in the presence of isoamylase (Table 4.1). For HAMS, starch recovery was independent of the presence of isoamylase. This outcome may be explained by the greater proportion of highly branched material (amylopectin) present in CCS than in HAMS. Thompson and Klucinec (1999) suggested that the presence of branched molecules in starch aqueous dispersions can hinder the formation of stable amylose aggregates that would otherwise form in absence of the branched molecules. For CCS, debranching led to greater molecular aggregation and consequent starch precipitation. For HAMS, molecular aggregation occurs independent of debranching by isoamylase, perhaps due to the higher ability of longer linear chains to associate.

For both CCS and HAMS, substantial amylopectin debranching resulted from the isoamylase treatment, as indicated by the absence of starch molecules eluting in the void volume region ($k' < 0.2$) of the SEC chromatograms (Fig. 4.2 and 4.3). For CCS, the greatest proportion of shorter chains was observed for the 2%R-20U sample; while for HAMS, the greatest proportion of shorter chains was observed for the 1%R-20U sample. Overall, for CCS and HAMS, the recovery of the short chains generated by isoamylase
debranching precipitates was greatest at the higher isoamylase level during rehydration, but differently affected by the starch concentration during rehydration.

During rehydration in the presence of isoamylase, two processes are occurring: 1) starch aggregation and 2) debranching. These two processes are in competition with each other, as shown in Figure 5.2. Because starch aggregation increases by increasing the starch concentration at rehydration, greatest extent of debranching would be expected for the sample rehydrated at 1%R in the presence of the higher isoamylase level (20U) for either CCS or HAMS. However, due to the lower tendency toward molecular aggregation of dispersed CCS, greater starch concentration at rehydration is required to enhance the recovery of the short chains generated from amylopectin debranching for CCS than for HAMS. For HAMS, the lower starch concentration at rehydration (1%R) is adequate to precipitate the longer short chains generated by isoamylase debranching.

For both CCS and HAMS, all the samples treated with isoamylase during rehydration had a higher weight percentage of amylose (molecules eluting in the chromatographic region I, Table 4.3) as compared to the samples rehydrated in the absence of isoamylase. Thus, starch debranching led to more and preferential amylose precipitation from dispersion. Formation of amylose-amylose double helices has been shown to be diminished in the presence of branched molecules (Klucinec and Thompson 1999). Debranching would be expected to increase amylose-amylose aggregation and consequent precipitation. However, preferential amylose precipitation from dispersion may also result from formation of stable mixed double helices of amylose and the short linear chains generated from amylopectin debranching.

The stereochemistry of the α(1→6) branch point is compatible with a stable double helix formed from two amylopectin chains connected through a branch point (Klucinec and Thompson 1999). The branch point may serve as a mechanism to keep to chains in proximity and bring them into proper register to form a double helix. Such a double helix would not contribute to amylose-amylopectin aggregation unless if part of a mixed crystallite. Formation of a double helix between two short linear chains generated from amylopectin debranching would be a kinetic disadvantage due to the additional requirement that the two chains must first align and register before a stable double helix
Figure 5.2 Competition of starch aggregation and debranching during rehydration of the ethanol precipitate in the presence of isoamylase (IA).
could form. If a short linear chain formed a mixed double helix with an amylose chain, the mixed double helix would be kinetically more favored than a double helix formed by two short linear chains. It would be also thermodynamically favored due to diminished mobility of the amylose chain and a diminished entropic contribution to “end effects”.

Moreover, analysis of the HPSEC spectra for fully debranched CCS and HAMS samples treated with isoamylase during rehydration indicated that for the shorter chain length of the linear chains generated by CCS debranching, there was a lower percentage recovery in the precipitate (Fig. 4.5 and 4.7, Table 4.3 and 4.4). The stability of a double helix increases with the length of the helix. Based on the increased likelihood for longer chains to form double helices of sufficient length to achieve stability, it is reasonable that the longer linear chains from CCS amylopectin and the long linear chains from HAMS amylopectin were more likely to participate in forming mixed double helices with amylose and possibly with other linear chains generated by starch debranching.
5.2 Effect of preparation conditions on the physical properties of the RS-containing starch preparations

5.2.1 Crystalline and helical order as determined by wide angle X-ray diffraction, solid-state $^{13}$C CP/MAS NMR and differential scanning calorimetry

Molecular order of starch materials can be investigated by wide angle X-ray diffraction (WXRD), solid-state $^{13}$C CP/MAS NMR and differential scanning calorimetry (DSC). WXRD can be used to investigate helical arrays that are sufficiently large and ordered to diffract X-rays. These regions are defined as crystalline. The part of the sample that does not diffract X-rays to give a distinct pattern is defined as amorphous. This amorphous material may still contain helical structures, as helices that are not associated with other helices, or as helical associations that are not sufficiently large or ordered to diffract X-rays in a distinct pattern. Regardless of the crystalline or amorphous nature by WXRD, the short range order, such as helical conformations, can be investigated by NMR. As shown in Fig. 5.3, even by the combination of these two techniques, the involvement of single or double-helical conformations in the crystalline and amorphous regions of the sample can not be discriminated.

By NMR, the C4 peak at ~82 ppm and the C1 peaks in the 95-103 ppm region have been shown to be most susceptible to conformational changes in starch (Gidley and Bociek, 1985). This is reasonable because these are the carbons associated with the main glycosidic linkage. The intensity of the peak at 82 ppm (Atichokudomchai et al. 2004, Bogracheva et al. 2001) and the intensity at 94-98 ppm have been associated with amorphous material (as defined by WXRD) (Gidley and Bociek 1985). Resonances in the 97-98.6 ppm ranges have been shown to be influenced by the starch sample preparation procedure (Paris et al. 2001). A C1 peak at 101 ppm has been associated with double helices (Gidley and Bociek 1985) while the C4 peak at ~82 ppm and the C1 peak at 103 ppm have been associated with single helices (Paris et al., 1999, Paris et al., 2001, Gidley and Bociek, 1985).
### Analytical techniques

<table>
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<td>Double-helical conformations</td>
<td>Crystalline – Amorphous</td>
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<tr>
<td>Non-helical conformations</td>
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Figure 5.3 Molecular order as determined by wide angle X-ray diffraction (WXRD) and solid-state $^{13}$C CP/MAS NMR. Single-helical conformations may be crystalline or amorphous, as can double-helical conformations.
The peak at ~82 ppm is a relative easier peak to analyze since it does not overlap as much with other related peaks. For this reason, the partial peak area (PPA) at ~82 ppm has been used (Atichokudomchai et al. 2004, Bogracheva et al. 2001, Evans and Thompson 2008) to characterize the helicity of starch samples. Bogracheva et al. (2001) suggested that for starch materials essentially free of single helices and with negligible signal intensity at 94-98 ppm, such as granular starches, the PPA at ~82 ppm could be used to estimate the proportion of non-double helical (NDH) material as follows:

\[
\frac{\text{PPA at 82 ppm of sample}}{\text{PPA at 82 ppm of amorphous reference}}
\]

NDH conformations would include single helices and non-helical conformations. Atichokudomchai et al. (2004) argued that in the absence of single helices the proportion of double helices in the sample may be calculated as:

\[
100 - \frac{\text{PPA at 82 ppm of sample}}{\text{PPA at 82 ppm of amorphous reference}}
\]

In the present study, DSC was used as a third analytical technique to investigate molecular order of the RS-containing starch preparations. DSC is a method of thermal analysis used to investigate endothermic and exothermic transitions of starch during heating and cooling at a controlled rate. For an endotherm to be produced by DSC, a sufficient quantity of helical or crystalline order would be required to dissociate within a sufficiently narrow temperature of range (Gidley and Bulpin 1992, Klucinec and Thompson 1999). The greater the number of glucose units involved in the helix formation, the greater the thermal energy required to overcome the enthalpic threshold to generate molecular motion. The temperature range for the endotherm of starch molecules dissociation should relate to the length of the melted helices.

### 5.2.2 Effect of rehydration of the ethanol precipitates on the physical properties

Of the ten samples analyzed by NMR, the ethanol-precipitated CCS and HAMS (NO R) had the sharpest C4 peak (~82 ppm) and the narrowest C1 peak (~103 ppm) (Fig. 4.11 and 4.12). Due to the sharp C4 peak (~82 ppm), the presence of mainly NDH conformations would be expected for these ethanol precipitates. Moreover, sharp peaks at ~103 ppm have been attributed to the presence of single helices (Gidley and Bociek
1985, Paris et al. 1999, Veregin et al. 1987). Therefore, the ethanol-precipitated CCS and HAMS had a greater proportion of single helical conformations than the other samples. For these ethanol precipitates, the amorphous pattern observed by WXRD diffraction (Fig. 4.8 and 4.9) indicates that the single helices in the ethanol precipitates were not sufficiently oriented in crystalline arrays to diffract X-rays and produce the V-type pattern (Biliaderis 1998). These findings are in agreement with Evans and Thompson (2008).

No endotherm was observed by DSC for CCS NO R and HAMS NO R (Fig. 4.13). For DSC analysis, the NO R samples were brought to approximately 33% solids by the addition of deionized water 30 minutes prior being placed in the DSC. It has been shown that starch single helices may change to double helices upon the 30 min hydration even at low moisture level by vapor phase solvent exchange (Le Bail et al. 1995, Saito et al. 1991). Although the CCS and HAMS NOR were brought to a water level much higher than what could be achieved by vapor phase solvent exchange, it is likely that rehydration of the starch samples led to formation of at least some double-helices. The melting of these helices may be occurring over such a broad temperature range that an endotherm is not readily apparent.

Although no endotherm was apparent, a large exotherm was observed at temperature >110°C for both the CCS and HAMS NO R (Fig. 4.13), indicating the formation of some sort of ordered association of starch molecules. As the CCS and HAMS NO R samples are heated in the DSC to temperatures >110°C, the molecules acquire sufficient energy to reorganize, perhaps as long double helices. Because no evidence of melting is observed on reheating, these ordered structures may not be coherently ordered, or they might melt above 180°C. Alternatively, the exotherm may result from hydration of single helices.

Analyses by WAXD (Le Bail et al. 1995) and by high resolution solid-state $^{13}$C NMR (Saito et al. 1991) showed that hydration of V-amylose by vapor phase solvent exchange results in the at least partial conversion to double helices organized as B-type polymorphs. In the present study, the RS-containing materials were prepared by rehydration of ethanol precipitates, by dispersion in water. Reorganization of the starch
molecules in a (2%) water dispersion did not lead to crystallization into B-type crystalline arrays for CCS nor for HAMS (Fig. 4.8 and 4.9).

Analysis of the NMR spectra indicated a less sharp C4 peak (~82 ppm) for the 2%R samples than for the NOR samples (Fig. 4.11 and 4.12). A less sharp C4 peak would indicate a lower proportion of NDH conformations, indicating a greater proportion of double helical conformations.

Greater double-helical order for the 2%R samples, as compared to the NOR samples, was also indicated by the DSC analysis. Contrary to the NOR samples, a broad endotherm was observed for the 2%R samples (Fig. 4.13 and 4.14). Because the temperature range for the endotherm of double helices dissociation should be related to helix length (Klucinec and Thompson 1999), the exceptionally broad endotherm observed for CCS 2%R and HAMS 2%R indicates that the samples were organized into a broad range of lengths of double helices.

Klucinec and Thompson (1999) investigated the retrogradation of CCS and HAMS fractions prepared by a two-stage aliphatic alcohol precipitation procedure by DSC. An endotherm at temperature higher ~140°C was observed in the amylose thermograms, while an endotherm at temperature lower than 100°C was observed in the amylopectin thermograms. Although the possibility can not be ruled out that a wide range of lengths in the amylose-amylose double helices accounts for the lack of a DSC endotherm at ~140°C, it is likely that amylose and amylopectin entanglement in aqueous dispersion limited the formation of long amylose-amylose double helices. The interference of intact amylopectin with amylose-amylose association would result in shorter amylose-amyllose double helices and in mixed double helices between amylose and amylopectin side chains. The amylose-amylopectin double helices would be lost over the same temperature range as amylopectin-amylopectin double helices (Klucinec and Thompson 1999). The broad endotherm at <140°C observed for CCS 2%R and HAMS 2%R suggests that amylose and amylopectin are mainly in the same phase in the starch precipitates.
5.2.3 Effect of rehydration in the presence of isoamylase on physical properties

A very weak B-type crystalline pattern was observed when the ethanol-precipitated CCS and HAMS were rehydrated in presence of isoamylase (2%R-20U) (Fig. 4.8 and 4.9). The crystallinity for the CCS 2%R-20U sample was more than double that for the HAMS 2%R-20U sample (Table 4.5). Nonetheless, for both samples, the weak pattern indicates that the crystallites in the samples were small and/or imperfectly packed.

Visual investigation of the NMR spectra for the CCS samples indicated that the spectrum of the 2%R-20U sample differed from the 2%R sample, mainly in the C1 and C4 peaks (Fig. 4.11). For the CCS 2%R-20U sample, the C1 peak was wider in the 101-103 ppm region, and the C4 peak was less distinct. Gidley and Bociek (1985) showed that the C1 peak of granular starches is wider in the 101-103 ppm region than for amorphous materials. The broader C1 peaks observed for granular starches were explained by the presence of multiple spectra signatures that can be attributed to the double helicity of the starch granules (Gidley and Bociek 1985; Paris et al. 1999). A less distinct C4 would indicate a lower proportion of NDH conformations and a greater proportion of double helices. Therefore, visual investigation of the CCS samples spectra, indicated a qualitatively greater proportion of double helical conformations for CCS 2%R-20U than for CCS 2%R, only some of which would be ordered in a crystalline array. No clear evidence for greater proportion of double helical conformations for HAMS 2%R-20U than for HAMS 2%R was observed by NMR.

By DSC, the greatest enthalpy of melting on initial heating (Table 4.8) was observed for the CCS 2%R-20U and HAMS 2%R-20U samples. The greater the enthalpy, the greater the double helix content of the sample (Gidley et al. 1995). An endothermic peak at ~140°C has been associated with retrogradation of amylose (Klucinec and Thompson 1999). Because the DSC endotherm for the CCS 2%R-20U and HAMS 2%R-20U samples was observed at <140°C (Fig. 4.13 and 4.14), it is likely that the aggregation of amylose and shorter linear chains generated by debranching during rehydration led to the formation of mixed double helices between amylose and the short linear chains. The formation of these mixed double helices would limit the formation of
long amylose-amylose double helices, thus explaining the loss of order being complete by 140°C.

The slightly greater percentage of crystallinity as observed by WXRD, the greater proportion of ordered material as observed by NMR, and the greater enthalpy of melting as observed by DSC for the 2%R-20U samples as compared to the 2%R samples indicate that greater double helical order resulted in the RS-containing starch materials as a consequence of debranching starch molecules during rehydration. Due to the weak B-type crystalline pattern as observed by WXRD, it may be suggested that small crystalline regions in the 2%R-20U starch samples are interspersed in a continuous amorphous (non-crystalline) phase, as shown in Figure 5.4. The amorphous (by WXRD) phase could contain a substantial amount of non-crystalline double helices. A general description of the CCS 2%R-20U and HAMS 2%R-20U samples would consist of double helices loosely arranged into aggregates with B-type packing geometry. The greater percentage of crystallinity observed for CCS 2%R-20U could result from the presence of bigger crystallites, more crystallites, and/or more prefect crystallites than in HAMS 2%R-20U.
Figure 5.4 Illustration of the proposed physical nature for the 2%R-20U starch samples. Only a portion of the sample is shown. Small crystalline regions are interspersed in a continuous amorphous (non-crystalline) phase.
5.2.4 Limitations of NMR data quantification

In the present study, peak fitting analysis was used to analyze the NMR spectra (Table 4.6). The PPA of the fitted peaks was determined with the intent of using the PPA of the C4 peak at ~82 ppm to determine the proportion of double helices in the samples according to Atichokudomchai et al. (2004).

The mathematical method used by Atichokudomchai et al. (2004) relies on the assumption that the amorphous reference is double helix-free. Evans and Thompson (2008) reported that by WXRD no indication of the presence of double helices was found for a sample obtained by ethanol precipitation of the branched fractions of HAMS. Based on that work, in the present study, ethanol-precipitated WCS from NaOH dispersion (WCS NOR) was considered for use as an amorphous reference. However, the WCS NOR could not be used as an amorphous reference to determine the proportion of double helices in the samples, as the PPA of the C4 peak for WCS NOR was lower than for the samples that had been dispersed and precipitated (Table 4.7). As noted, the branched material used by Evans and Thompson (2008) to prepare the amorphous reference was from HAMS. The branched fraction of HAMS has a lower degree of branching, higher average chain length, and a larger proportion of long chains than of WCS (Klucinec and Thompson 1998, Takeda et al. 1993, Takeda et al. 1992a, b). It is likely that the lower proportion of longer linear chains in WCS would have hindered the formation of stable single helices, which would account for the smaller PPA at ~82 ppm.

Despite the problems associated with calculation of the proportion of double or single helices in the starch materials from the PPA of the C4 peak, visual investigation of the NMR spectra was still prove useful to gain insight into the overall helical conformation in the starch samples.
5.3 Influence of preparation conditions on the enzyme digestibility of the RS-containing materials

5.3.1 Effect of rehydration in the absence of isoamylase of the ethanol precipitates on the enzyme susceptibility

Rehydration of the ethanol precipitate did not enhance the RS content for the CCS samples, but it did somewhat for the HAMS samples (Table 4.2). For non-granular starch materials, resistance to enzymatic digestion has been attributed to both crystalline and non-crystalline double helices (Gidley et al. 1995; Jane and Robyt 1984). The difference in the enzyme susceptibility for the rehydrated ethanol-precipitated CCS and HAMS could be explained by differences in the helicity and/or crystallinity of the samples. However, physical characterization of the CCS 2%R and HAMS 2%R samples suggested the presence of double helices with a broad range of lengths, in an amorphous conformation for both samples. This outcome indicates that the covalent molecular structures of the samples may play an important role in the enzyme susceptibility.

The RS content for starch materials prepared from precipitation from sodium hydroxide dispersion has been shown to increase with the percentage of linear fraction in the precipitate (Evans and Thompson 2008). Similarly, it can be argued that the greater RS content of the samples obtained by rehydration of the ethanol-precipitated HAMS resulted from a higher proportion of amylose, as observed by SEC (Fig. 4.2 and 4.3) and HPSEC (Table 4.3 and 4.4). However, chromatograms of debranched ERS from HAMS 1%R and 2%R (Fig. 4.17) indicated that the ERS had a wide range of linear chains with dp< 200. Thus, no evidence for the presence of intact amylose in the ERS from HAMS 1%R and 2%R was found. This outcome suggests that mixed amylose-amylopectin, and possibly amylopectin-amylopectin, double helices contributed to the resistance to digestion for the samples obtained by rehydrating the ethanol-precipitated HAMS.

Because of the higher amylose content and the higher proportion of longer amylopectin chains in HAMS, a higher proportion of longer double helices should be present in the samples obtained by rehydrating the ethanol-precipitated HAMS than CCS. The longer double helices in the HAMS samples would be more resistant to enzymatic
digestion than the shorter double helices in the CCS samples, thus accounting for the difference in RS content between the samples.

Substantial amounts of RS from essentially amorphous HAMS starch materials have been previously shown by Htoon et al. (2008). Work from the same group (Lopez-Rubio et al. 2008) indicated that the enzyme-resistance in amorphous HAMS starch materials may be enhanced during the digestion of the materials, as a result of amylose rearrangement into crystalline double helices. In the present study, because the physical properties of the ERS from the HAMS have not been investigated, the possibility that the double helical order in the HAMS samples was enhanced during the digestion for the RS determination could not be excluded.

5.3.2 Effect of rehydration in the presence of isoamylase on the enzyme susceptibility

In this study, debranching of starch molecules has been effective in generating RS even from starting materials that have essentially no RS character, such as the samples obtained by rehydrating the ethanol-precipitated CCS (Table 4.2). This outcome strongly suggests active involvement of the linear chains generated by debranching in RS formation.

Debranching of the rehydrated ethanol precipitates favored the alignment of the starch molecules, leading to development of some crystallinity (Fig. 4.8 and 4.9). For all samples examined, the proportion of crystallinity (Table 4.5) was much smaller than the RS values (Table 4.1). The material making up the RS in the samples obtained by rehydrating the ethanol precipitates in the presence of isoamylase is therefore only partially crystalline, and it likely contains considerable amounts of amorphous material.

The RS content of the CCS samples doubled by doubling the isoamylase level at rehydration, regardless of the starch concentration at rehydration (Table 4.2). The lower enzyme susceptibility of the CCS samples obtained by doubling the isoamylase content at rehydration can be partially explained by the higher proportion of crystallinity of the samples, as seen by WAXD (Table 4.4). Double helices in the CCS samples would contribute to RS only if organized in a B-type crystalline array. Because the crystallinity
of CCS samples doubled by doubling the isoamylase level at rehydration, an even more complete debranching of CCS molecules may be needed to maximize the RS content.

Contrary to the behavior of CCS, the RS content of the HAMS samples decreased by doubling the starch concentration at rehydration, and it was independent of the isoamylase level at rehydration (Table 4.2). In this study, substantial aggregation of intact HAMS molecules has been shown to occur even in absence of the isoamylase at rehydration (Table 4.1). As shown in Fig. 5.2, aggregation of the intact HAMS starch molecules would hinder the extent of debranching. This interference would remain even when the isoamylase level at rehydration is doubled. Because starch aggregation decreases by decreasing the starch concentration at rehydration, less rapid aggregation of HAMS molecules might be needed to maximize the RS content for two reasons: 1) rapid retrogradation interferes with ordering structures; 2) rapid retrogradation interferes with debranching.

The chromatograms of debranched ERS from the RS-containing starch materials (Fig. 4.15 and 4.17) were substantially different from the chromatograms of debranched ERS from granular HAMS (Fig. 4.19). Although starch debranching led to almost complete amylose precipitation in the RS-containing starch preparations, essentially no amylose (molecules eluting in the chromatographic region I) was observed in the chromatograms of debranched ERS (Fig. 4.16 and 4.18). On the other hand, ERS from HAMS was about 17% of the initial amylose (Table 4.10). This outcome indicates that intact amylose is partly digested by pancreatic α-amylase during the RS assay in the RS-containing starch materials, but not as much in digested granular HAMS.

For the RS-containing starch preparations, the chain length of ERS ranged from dp 10 to ~100 (Fig. 4.15 and 4.17) with an approximate relative maximum at dp 20-30. This profile is in general agreement with previous studies of digestion of retrograded starches (Berry 1986, Gidley et al. 1995, Leloup et al. 1992). Since the minimum chain length required for stable double helix formation is 10 dp (Gidley and Bulpin 1987), the absence of chains <10 dp in the chain length profile of ERS would support the proposed origin of RS in non-granular starch materials as double helical conformation.

However, for granular HAMS, chains with dp > 200, likely intact chains from amylose and intermediate material (Klucinec and Thompson 1998), were observed in the
chain length profile of ERS. Rees (2008) investigated the pattern of granular HAMS digestion by pancreatic α-amylase during the RS assay by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Most granules appeared to be attacked from within, with partially digested granules appearing intact from the exterior. The presence of intact amylose and intermediate material in ERS from HAMS would be consistent with these molecules being present in the remaining parts of the granules.

5.4 Effect of particle size and thermal treatment on RS content

5.4.1 Effect of particle size and thermal treatment on the RS content of the RS-containing starch preparations

Of the seven preparation procedures followed to generate RS-containing starch materials from CCS, the highest proportion of RS determined as percentage of the starting material was obtained by rehydrating the ethanol-precipitated CCS at 2% in the presence of 20U isoamylase (sample 2%R-20U) (Fig 4.1). For HAMS, the proportion of RS determined as percentage of the starting material, did not vary greatly among the samples obtained by rehydrating the ethanol precipitate in the presence of isoamylase (Fig. 4.1). Therefore, to compare the preparation procedures directly, the 2%R-20U was chosen for HAMS as well. For these two samples the effect of particle size and thermal treatment of RS content was investigated.

For both samples, the proportion of RS decreased as particle size decreased (Fig. 4.21). Smaller particles have a higher surface area. A greater surface area would result in a greater proportion of the starch material available to the enzyme, thus increasing the rate of starch hydrolysis. In other words, a smaller surface area could be seen as a barrier to physical accessibility to the otherwise digestible starch. Physically inaccessible starch has been classified as type 1 RS (Englyst et al. 1992). Therefore, the proportion of type 1 RS in the RS-containing starch materials investigated would decrease as particle size decreased.

Beside the type 1 RS, enzyme resistance of the RS-containing starch materials was attributed by the chemical and physical characterization to the reassociation of starch
molecules subsequent to rehydration and debranching of the ethanol precipitates. Although type 3 RS is usually attributed to reassociation of starch molecules subsequent to cooking (Englyst et al. 1992), the terminology “type 3 RS” could be used to describe the RS generated by reassociation of solvent-dispersed starch molecules as in this study. Thus, the RS-containing starch materials could be considered as a combination of type 1 and type 3 RS. Theoretically, an infinitesimal particle would have no type 1 RS. Assuming that the proportion of type 3 RS for the RS-containing starch material is independent of the particle size, the proportion of type 3 RS could be estimated from the RS when particle size approached zero. Based on this thinking, the proportion of type 3 RS was estimated to be ~13% for CCS 2%R-20U and ~19% for HAMS 2%R-20U, by using the regression equation in Fig. 4.21.

For each RS-containing material, the shape of the ERS chain length profile was independent of particle size (Fig. 4.22 and 4.24). Therefore, no evidence was found to indicate that the proportion of type 1 RS influenced the chemical nature of the ERS. This outcome might be explained by the pattern of digestion shown in Figure 5.5. The physical nature of the 2%R-20U starch preparations consists of double helical chains arranged into aggregates with B-type packing geometry interdispersed in a continuous amorphous phase. The amorphous regions would be expected to be preferentially hydrolyzed by the pancreatic α-amylase, independently of the particle size. However, because of the higher surface area, the amorphous regions of the smaller particles would be more readily accessible, and would be hydrolyzed at a higher rate than the amorphous regions of the bigger particles. Because of the similarity of the ERS chain length profile for particles with different size, it is likely that by the end of the 16 h digestion the amorphous regions for both particle sizes were completely hydrolyzed. That end suggests that the difference in the RS content among samples with different particle size would arise from differences in the extent of hydrolysis of the partially crystalline regions of the particle.
After 16 h pancreatic α-amylase digestion

Figure 5.5 Pattern of digestion suggested for RS-containing starch preparations with different particle size.
Black circles represent the partially crystalline regions of the sample; grey areas represent the amorphous regions of the sample.
The effect of a thermal treatment on the RS content of the CCS 2%R-20U and HAMS 2%R-20U samples with different particle size was also investigated. Results indicated that the preparation procedure used in the current study successfully generated RS-containing starch materials that had a similar RS content level even following a 30 min boiling treatment in excess water (Fig. 4.21). This outcome was particularly interesting for the RS-containing starch material prepared from CCS, since no RS is observed in CCS following thermal treatment.

The proportion of RS following thermal treatment was independent of particle size, and was similar to the putative type 3 RS estimated for CCS 2%R-20U and HAMS 2%R-20U (Fig. 4.21). It is likely that the thermal treatment caused swelling of the starch particles, at least partially reducing the physical hindrance that may account for the proportion of type 1 RS observed prior to the thermal treatment. As a result, the thermal treatment drastically reduced the type 1 RS in the samples.

A significant portion of the DSC endotherm for CCS 2%R-20U and HAMS 2%R-20U, particle size <180 μm, was at <100°C (Fig. 4.13 and 4.14). Although the thermal treatment prior to RS determination was done at lower solids content than the DSC analysis, the DSC analysis was still considered “excess water” (Biliaderis et al. 1986a, b). Thus it can be expected that a good portion of the double helices that contributed to type 3 RS in CCS 2%R-20U and HAMS 2%R-20U would be lost as a consequence of the thermal treatment. The temperature range for the loss of double helices should relate to helix length. The longer the double helix, the greater the thermal energy required to unwind it. During the thermal treatment prior to the RS determination, the shorter double helices would unfold. It would be therefore expected that the RS thermally treated prior to the RS determination would have a greater proportion of longer double helices (those stable at 100°C) than RS, with some likely due to formation during the thermal treatment. This understanding would be in agreement with the greater proportion of longer chains shown by the chromatograms of debranched ERS thermally treated prior to the RS determination (TT-ERS) as compared to ERS (Fig. 4.22 and 4.24).

Use of heating/cooling cycles has been shown to lead to increased levels of type 3 RS, by enhancing the double helicity and/or crystallinity of the starch materials (reviewed in Thompson (2000b)). Rees (2008) proposed that resistant material is being formed.
during the 30 min boiling/digestion treatment of the total dietary fiber (TDF) assay, due to helix extension caused by initial enzyme hydrolysis that allowed chain motion. In the present study, the thermal treatment (30 min boiling) prior to RS determination was done in absence of the enzyme. Eerlingen et al. (1993) reported that at 100°C, the propagation rate of amylose crystals in gelatinized starch was favored even though the nucleation rate was rather limited. Once a few nuclei were formed, they were extensively propagated. In the present work, the thermal treatment prior RS determination would lead to mobility of the less stable chains, which then could use remaining helices for nucleation, and realign and form more highly ordered and stable structures. The structures that did not melt during the thermal treatment, i.e. the longer double helices, would act as templates for an at least partial realignment of the newly mobile chains, leading to enhancement of more highly ordered structures. The enhancement of ordered structures could either happen during the thermal treatment or during cooling after the treatment.

This thinking has been employed to suggest that increases in order might even occur without heating, during the RS determination assay, as suggested by Lopez-Rubio et al. (2008).

Evidence for potential enhancement of ordered structures upon the thermal treatment prior to RS determination may be found in the DSC scans. Boltz and Thompson (1999) investigated the behavior of high-amylose maize starches during cooling and subsequent reheating by DSC. On cooling, starch samples showed two endotherms, which they attributed to amylose-lipid association (~75°C) and amylose-amyllose associations (~30°C). Upon reheating, starch samples showed an endotherm, which they attributed to melting of ordered amylose. In the present study, for cooling of HAMS 2%R-20U, the exotherm at ~30°C was sharper and at higher temperature than the exotherm observed for the non-debranched HAMS samples (Fig. 4.14). For CCS 2%R-20U, an exotherm at ~30°C was observed, but no exotherm was observed for the non-debranched CCS samples (Fig. 4.13). This outcome indicates a greater starch molecular association upon cooling for the debranched RS-containing starch materials, which may result from the self-association of short linear chains generated by starch debranching or the mixed association of the short linear chains with the amylose, in addition to the amylose-amylose association, upon cooling.
Moreover, the endotherm for reheating of the CCS 2%R-20U and HAMS 2%R-20U samples shifted toward higher temperatures as compared to the endotherm on initial heating (Fig. 4.13 and 4.14). This outcome indicates that the association of the starch molecules upon cooling lead to the formation of a higher proportion of longer double helices then the one present in the sample prior to the heating and subsequent cooling by DSC. Although the thermal treatment prior to RS determination was done at different conditions (lower solid content, lower heating temperature, longer holding time, faster cooling) than the DSC scans, the thermal treatment prior to RS determination could lead to formation of longer double helices in the samples. These double helices would contribute to the RS thermally treated prior to the RS determination.

5.4.2 Effect of thermal treatment on the RS content of granular HAMS

For granular HAMS the chain length profile of the fully debranched TT-ERS differed from the profile of ERS (Fig. 4.23), also changed, but in a different way than for the RS-containing starch preparations. Linear chains with dp> 200, considered to be chains from intact amylose and intermediate material (Klucinec and Thompson 1998), were not observed in the profile of TT-ERS (Fig. 4.23). This outcome indicates that amylose and intermediate material in HAMS were more susceptible to enzyme digestion during the RS determination following thermal treatment. Nevertheless, more intermediate chains (fraction II, Fig. 4.23) remained in the TT-RS. This outcome may indicate that the longer chains (fraction I) in TT-RS from HMAS were less completely helical and, therefore more susceptible to reduction in length due to pancreatic α-amylase digestion, than the intermediate chains.

5.5 Digestion time course

The official method for in vitro determination of RS (AOAC Method 2002.02), directly analyzes the starch recovered by precipitation after 16 hours digestion with pancreatic α-amylase. In the present study, the digestion time was extended beyond the stipulated 16 hours. Although digestion was essentially complete by the end of the 16
hours for HMT (as also seen by Rees, 2008), for HAMS, CCS 2%R-20U and HAMS 2%R-20U hydrolysis continued beyond 16 hours. This outcome suggests that the RS from HAMS, CCS 2%R-20U and HAMS 2%R-20U has a residual susceptibility to pancreatic α-amylase digestion. Therefore, for these materials, the RS content determined by the official method for in vitro determination of RS is due to the kinetics of pancreatic α-amylase digestion and not to a digestion limit being reached. Because the 16 hours digestion time has been validated by comparison against in vivo resistance as determined in human subjects, it can be assumed that for these materials a limit in digestion would not be reached in vivo in the conditions of the small intestine. The implication is that the kinetics of digestion in the small intestine is sufficiently slow that gut transport would lead to passage into the colon before a limit to starch digestion by pancreatic α-amylase could be reached. The RS from these samples may be more susceptible to hydrolysis in the colon, and this increased susceptibility would thus favor hydrolysis in the proximal colon.

A plot of the digestion timecourse of all the granular and non-granular starch samples showed a fast digestion in the beginning that slowed down with increasing digestion time (Fig. 4.25), as seen previously (Brumovsky and Thompson 2001, Evans and Thompson 2008, Planchot et al. 1997, Rees 2008). Two substrates with different digestion rates have been previously claimed for both granular and non-granular starch (Englyst et al. 1992, Evans and Thompson 2008, Rees 2008). Based on the understanding that starch digestion is characterized by the presence of two components, kinetic analysis was performed by utilizing a 5-parameter, double-exponential model (Eq. 1 in section 3.3.3.2) as in Rees (2008).

The double-exponential decay model describes HAMS and HMT as the sum of two distinct starch substrates (S₁ and S₂) which are hydrolyzed at two different rates (k₁ and k₂) and a portion of starch that is not digested by pancreatic α-amylase (SND). The triple-exponential decay model describes the CCS 2%R-20U and HAMS 2%R-20U samples as the sum of three distinct starch substrates (S₁a, S₁ and S₂) which are hydrolyzed at three different rates (k₁a, k₁ and k₂). Therefore, according to these models, when the digestion time is extended beyond the stipulated 16 hours, digestion by pancreatic α-amylase of the RS from the CCS 2%R-20U and HAMS 2%R-20U samples
would eventually be complete, while digestion of the RS from HAMS and HMT would reach a limit \((S_{ND})\).

For HMT, the limit to pancreatic \(\alpha\)-amylase digestion \((S_{ND} = 46.3\%, \text{ Table 4.12})\) was similar to the RS content \((54.1\%, \text{ Fig. 4.25})\), while for HAMS, the limit to pancreatic \(\alpha\)-amylase digestion \((S_{ND} = 18.7\%, \text{ Table 4.12})\) was less than half of the RS content \((47.2\%, \text{ Fig. 4.25})\). Similar results had been obtained by Rees (2008). This outcome suggests that the RS from HMT has very little residual susceptibility to pancreatic \(\alpha\)-amylase digestion after 16 hours, while more than half the RS from HAMS is susceptible to further pancreatic \(\alpha\)-amylase digestion. Rees (2008) suggested that during the heat-moisture treatment, a portion of the structures that comprised the slowly digestible substrate \((S_2)\) in HAMS could become more stable, through increased double-helical associations or improvement of alignment of double-helices, so that more starch becomes inaccessible to pancreatic \(\alpha\)-amylase digestion in HMT.

Kinetic analysis of the digestion time course showed that the reaction rate constant for \(S_{1a}\) observed for CCS 2%R-20U and HAMS 2%R-20U was much higher than the reaction rate constants for \(S_1\) and \(S_2\) (Table 4.12). The rate constants for \(S_1\) and \(S_2\) for CCS 2%R-20U and HAMS 2%R-20U were similar to the rate constants for \(S_1\) and \(S_2\) for HAMS and HMT (Table 4.12). Because a high reaction rate constant indicates a high susceptibility to digestion, \(S_{1a}\) would be exceptionally susceptible to pancreatic \(\alpha\)-amylase digestion, a type of substrate not present in the granular starches investigated.

Evans and Thompson (2008) suggested that for ethanol-precipitated non-granular starches (like the No R samples in the present work), the substrate digested more rapidly might be composed of amorphous material, while the substrate digested more slowly might contain both amorphous and crystalline structures. In the present work with the CCS 2%R-20U and HAMS 2%R-20U samples, the proposed physical nature is illustrated in Fig. 5.6. In this model, small crystalline regions are interdispersed in a continuous amorphous (non-crystalline) phase. \(S_{1a}\) might be composed of the amorphous and largely non-helical phase; while \(S_1\) might be a combination of amorphous (both helical and non-helical) and crystalline material. \(S_2\) would be mainly composed of crystalline material.
Figure 5.6 Illustration of the proposed physical nature for the 2%R-20U starch samples. The ovals highlight three regions according to the three different types of substrates ($S_{1a}$, $S_1$ and $S_2$).
5.6 Extracellular amylolytic activity of pure cultures of *Bifidobacterium* strains

5.6.1 Soluble-starch agar plate assay

A soluble-starch-agar plate assay was used by Wang et al. (1999) and Ryan et al. (2006) to compare the amylolytic activities of bacterial strains, including bifidobacterium strains. A different inoculum technique was used in these two studies. Wang et al. (1999) transferred aliquots of the tested cultures to a sterile paper disk placed on the surface of the agar plate, while Ryan et al. (2006) transferred aliquots of the tested cultures to individual wells formed on the surface of the agar plate. In the present study, both inoculum techniques were used for the development of the assay (Table 4.13). The technique of forming wells in the agar plate was the most straightforward inoculum technique. With this inoculum technique, the lowest variation was found for the 10 μl inoculums level. Thus, the technique of forming wells in the agar plate with inoculation levels of 10 μl was used in the following studies.

Following incubation of the bacterial cultures in the wells of the agar plates, the plates were stained with an iodine solution. The presence of unstained regions around the paper disk or the well, referred to as unstained zones, would result as a consequence of soluble starch hydrolysis. In their study, Wang et al. (1999) and Ryan et al. (2006) attributed the presence of unstained zones to starch degradation by bacterial amylolysis. They did not elaborate on whether the starch degradation resulted from the action of the bacterial cells or of the bacterial extracellular enzymes diffusing through the agar gel. However, because bifidobacteria cells are immobile and can not diffuse through the agar gel, in the present study the formation of the unstained zones will be attributed to starch degradation by the action of the bacterial extracellular amylolytic enzymes. The amylolytic enzymes would diffuse radially from the disk or the well on the agar plate, progressively degrading the starch molecules. From the data of Table 4.13, only *B. animalis* ssp. *lactis* Lafti B94 and *B. pseudolongum* ATCC 25526 synthesize extracellular amylolytic enzymes capable of degrading soluble starch.
The absence of unstained zones, as for *B. animalis ssp. lactis* DSMZ 10140, indicates that the bacterial strain tested did not synthesize extracellular amylolytic enzymes capable of degrading soluble starch under the conditions of the test. However, the tested bacterial strain could still be capable of degrading soluble starch by action of membrane-associated amylolytic enzymes. This assay would not allow membrane-associated hydrolysis to be evaluated.

5.6.2 Native starch and ERS agar plate assays

The question of which bacterial species are capable of degrading starch *in vitro* has been previously addressed (Bird et al. 2000, Crittenden et al. 2001, Fassler et al. 2006, Lesmes et al. 2008, Ryan et al. 2006, Wang et al. 1999b, Wronkowska et al. 2006). In most studies, starch granules have been added to a culture medium and then autoclaved (Crittenden et al. 2001, Ryan et al. 2006, Wang et al. 1999b). Autoclaving of starch granules leads to an at least partial disruption of the granular structure. In the present study, granular HAMS was added to the culture medium following autoclaving, maintaining the integrity of the native granular structure. Instead of heat treatment for sterilization, HAMS was washed with 80% ethanol and exposed to UV light for 30 min prior being added to the sterile medium. The treatment was successful in avoiding the proliferation of microbial contamination naturally present on HAMS during the assay, without modifying the physical structure of HAMS.

Iodine staining of the HAMS-containing agar plates following incubation was attempted with both iodine solution and iodine vapor. When the iodine solution was added to the surface of the plate, no zones could be distinguished from the dark blue background. However, when the plate was exposed to iodine vapor, lighter regions around the wells, referred to as light zones, could be distinguished from the light brown background for *B. animalis ssp. lactis* Lafti B94 and *B. pseudolongum* ATCC 25526. This outcome indicates that the extracellular amylolytic enzymes synthesized by *B. animalis ssp. lactis* Lafti B94 and *B. pseudolongum* ATCC 25526 could at least partially degrade HAMS granules.
Because the zones formed on the HAMS-containing agar plates were not clear, it is likely that the HAMS granules were not completely degraded by the bacterial extracellular enzymes during the incubation time. The undigested portions of the granules would still maintain the ability to bind with iodine. It appears that the iodine solution would stain the HAMS granules and the undigested portions of the granules similarly, making it impossible to visually distinguish the presence of eventual zones. On the other hand, the iodine vapor would stain the intact HAMS granules a slightly darker color than the digested portions of the granules, leading to the formation of not completely clear zones, but zones that are lighter than the gel before inoculation.

The agar plate assay was also utilized to test whether ERS isolated from HAMS by the official method for in vitro RS determination (AOAC 2002.02, AACC 32-40) could be at least partially degraded by extracellular amylolytic enzymes synthesized by *B. animalis ssp. lactis* Lafti B94 and *B. pseudolongum* ATCC 25526. When the ERS from HAMS-containing agar plates where exposed to iodine vapor following incubation, no zone could be distinguished from the stained background (data not presented). It is likely that the iodine vapor would stain the ERS and the partially degraded ERS similarly making it impossible to visually distinguish the presence of eventual zones. Therefore, the absence of zones was interpreted as a limitation of the methodology rather than a lack of ERS degradation.

5.6.3 Agar plate assay to estimate activity in a pancreatic α-amylase solution

When only pancreatic α-amylase solution was placed in the wells, the radius of the zones on both soluble starch and granular HAMS-containing agar plates increased with increasing the activity of the pancreatic α-amylase inoculated (Fig. 4.28). Therefore, with respect a particular starch substrate, the radius of the zone depends directly on the activity of pancreatic α-amylase inoculated. For the same enzyme activity, the unstained zone formed on soluble starch-containing agar plates was of smaller radius than the light zone formed on granular HAMS-containing agar plates.

The difference in size of the zones formed on agar plates with different starches may result from differences in the diffusion rate of the enzyme. Differences in the
enzyme diffusion rate may be due to the different structure of the polymer matrix through which the enzyme is diffusing and to the affinity of the enzyme for the starches tested.

For the soluble starch-containing agar, the polymer matrix is a continuous network composed by a mixture of agarose and agaropectin in which the soluble starch is dispersed. The enzyme will diffuse through this continuous network, progressively hydrolyzing the starch molecules. For the granular HAMS-containing agar, the polymer matrix is more discontinuous: HAMS granules will be present in an agarose and agaropectin network. The enzyme will diffuse through the agarose and agaropectin network, and it will adsorb on and into the HAMS granules for subsequent starch hydrolysis. The diffusion rate of pancreatic $\alpha$-amylase might be more retarded in the soluble starch-containing agar, as it associates with the soluble starch until hydrolysis is complete. For the HAMS-containing agar, there would be less retardation, due to the less complete hydrolysis (unless the enzyme became trapped within the granule).

### 5.6.4 Agar plate assay for extracellular activity for strains of Bifidobacterium

Little is known about bifidobacterial enzymes involved in starch breakdown. It is important to note that the nature of the activity of the vast majority of extracellular amylolytic enzymes synthesized by bifidobacterium strains is still unknown. Although three extracellular amylolytic enzymes have been isolated and purified from *Bifidobacterium* species (Ji et al. 1992, Lee et al. 1997, O'Connel Motherway et al. 2008), the activity of these enzymes has not yet been characterized. Moreover, it may be possible that multiple amylolytic extracellular enzymes may be involved in starch degradation for a single bifidobacterial strain.

The radius of the zones formed on the starch-containing agar plates depends on the diffusion rate of the extracellular amylolytic enzymes. The diffusion rate of the extracellular amylolytic enzymes may depend upon several factors, such as the substrate-enzyme affinity, the size of the enzyme, and the rate of the catalytic event. The radii of the zones formed on the starch-containing agar plates may be affected by these factors, and cannot be simply interpreted strictly as a function of the bacterial amylolytic activity.
Nonetheless, it seems reasonable to interpret the radii of the zones as estimates of extracellular amylolytic activity of bifidobacterium strains.

*B. choerinum* ATCC 27686, *B. infantis* ATCC 15697, and *B. pseudolongum* ATCC 25526, formed light zones on HAMS-containing agar plates with the greatest radii among the 31 strains that exhibited extracellular amylolytic activity (Table 4.15). Because these three strains had the greatest activity, were selected to further investigate growth on prepared putative RS per se (Section 5.9).

Two other strains, *B. animalis* subsp. *lactis* DSMZ 10140 and *B. animalis* subsp. *lactis* BB12, were also selected for further studies to explore the possibility of induction of amylolytic activity. The genome of *B. animalis* subsp. *lactis* DSMZ 10140 is known. Genes sequencing suggests that this strain has the potential to utilize a wide range of complex carbohydrates (Barrangou et al. 2009). However, by utilizing the zone on agar plate assay, no unstained zones were observed on the soluble starch-containing agar plates, indicating that *B. animalis* subsp. *lactis* DSMZ 10140 does not synthesize extracellular amylolytic enzymes. Nevertheless, because of the genome information, the potential for carbohydrate metabolism of *B. animalis* subsp. *lactis* DSMZ 10140 justified further studies (Section 5.8.4).

When administrated live in adequate amounts, some bifidobacterium strains confer health-promoting effects for the host, including prevention of diarrhea, anticarcinogenity, and inhibitory activity against pathogens (Gibson and Roberfroid 1995, Parvez et al. 2006). For their health-promoting effects, these bifidobacterium strains are recognized as being probiotic. *B. animalis* subsp. *lactis* BB12 is among the most extensively studied probiotic strain of bifidobacteria (Briczinski 2007). By utilizing the zone on agar plate assay, *B. animalis* subsp. *lactis* BB12 formed intermediate-sized unstained zones on soluble starch, and light zones on HAMS-containing agar plates (Table 4.15). This strain was also selected for further studies (Section 5.8.4).
5.7 Soluble extracellular amylolytic enzyme activity synthesized by selected strains of *Bifidobacterium* in presence of different carbohydrate substrates

Synthesis of extracellular amylolytic enzymes may be induced (i.e., initiated or enhanced) in response to a deficiency in a preferred substrate. The present experiment was designed to test whether a greater synthesis of soluble extracellular amylolytic enzymes would result by growing the five selected *Bifidobacterium* strains on increasingly challenging carbohydrate sources (in the order glucose < soluble starch < HAMS < ERS from HAMS).

The selected *Bifidobacterium* strains (described in the previous section) were grown in the test-starch-containing broth. Cell-free supernatant (CFS) was obtained by removing the bacterial cells after growth in glucose, soluble-starch, HAMS or ERS from HAMS. The CFS was placed in the well formed on the soluble starch-containing agar plates, and incubated at 37°C. The radius of unstained zones on the soluble starch-containing agar plates following incubation was used as an indication of the activity of extracellular amylolytic enzymes in the CFS. It was hypothesized that the more challenging to degrade the carbohydrate substrate, the greater the radius of the unstained zones would be, indicating a greater induction of synthesis of extracellular amylolytic enzymes.

For *B. animalis* subsp. *lactis* DSMZ 10140, the lack of unstained zones on the soluble starch-containing agar plate (Table 4.16) indicated that no soluble extracellular amylolytic enzymes was synthesized when the strain was grown on glucose, soluble starch or HAMS. For *B. pseudolongum* ATCC 25526, there was evidence of greater synthesis of soluble extracellular amylolytic enzymes when grown on HAMS as compared to soluble starch or glucose. Thus, it seems that for *B. pseudolongum* ATCC 25526, growing the strain on a more challenging carbohydrate source induced greater synthesis of soluble extracellular amylolytic enzymes.
For the other three strains analyzed, no evidence for induction of extracellular amylolytic enzymes in the presence of more challenging to degrade carbohydrate sources was observed (Table 4.16). For *B. choerinum* ATCC 27686, activity was evident for growth on all substrates, and no clear trend was observed. For *B. animalis* subsp. *lactis* BB12 and *B. infantis* ATCC 15697, the unstained zones were too small to be accurately measured; thus the question of whether induction of extracellular amylolytic enzymes may result is still an unresolved question for these two strains.

For all strains that exhibited extracellular amylolytic activity, the radii of the unstained zones formed on the soluble starch-containing agar were greater when the unfiltered culture was placed in the wells as compared to when the CFS was placed in the wells (Table 4.17). This outcome indicates that the cells in the well are actively producing extracellular amylolytic enzymes over the incubation time in the soluble starch-containing agar plates.

When ERS from HAMS was used as carbohydrate substrate, the CFS of all 5 strains tested, including *B. animalis* subsp. *lactis* DSMZ 10140, formed unstained zones on the soluble starch-containing agar plates. Moreover, the filtered media from the uninoculated ERS from HAMS-containing broth (the putative negative control) also formed unstained zones on the soluble starch-containing agar plates (Table 4.16). These results indicated the presence of soluble amylolytic enzymes associated with the ERS from HAMS.

Alpha-amylases are involved in the hydrolysis of granular starch in germinating cereal kernels. It might be argued that the amylolytic activity associated with the ERS from HAMS may result from the presence of these enzymes in native HAMS granules. However, uninoculated HAMS-containing broth did not form unstained zones on the soluble starch-containing agar plates (Table 4.16). Thus, the residual amylolytic activity associated with the ERS from HAMS results from the ERS preparation. Because the main amylolytic enzyme involved in the ERS preparation is pancreatic α-amylase, it is likely that the residual amylolytic activity results from the presence of residual pancreatic α-amylase associated with the ERS. Because a primary objective of the present thesis was to explore whether bifidobacterium strains could utilize RS, this finding fundamentally shifted the interest of our investigation, and further experiments were designed in order to
investigate whether the presence of residual pancreatic α-amylase associated with the ERS might contribute to the continued digestion of RS by the bifidobacteria. As a result, the experiment was not replicated.

5.8 Residual amylolytic activity associated with starch resistant to the 16 hour digestion

5.8.1 Reducing the residual amylolytic activity

ERS was obtained by ethanol precipitation of the HAMS resistant to 16 hours pancreatic α-amylase digestion, as described in the official method for in vitro RS determination (AOAC 2002.02, AACC 32-40) (see section 3.3.4.1). In the description of the RS determination procedure, the stated purpose for the addition of ethanol in the RS determination is to terminate the starch hydrolysis (McCleary and Monaghan 2002). Although the authors did not elaborate on the mechanism that would lead to the termination of the starch hydrolysis, one may suggest that the reaction would terminate as a consequence of denaturation of pancreatic α-amylase.

Because the hydrophobicity of ethanol is low, the addition of ethanol to the maleate buffer would not be expected to make the solvent more non-polar. The denaturation of the pancreatic α-amylase is most likely due to dehydration. Ethanol molecules may disrupt the water hydration layer at the protein surface. The protein would change conformation to minimize its energy under the new conditions. In its new conformation, the pancreatic α-amylase would not be active on starch. Results in the present study (Table 4.18) indicate that although the addition of ethanol in the RS determination assay may inactivate the pancreatic α-amylase, the denaturation of the enzyme is at least partially reversible when the enzyme is redispersed in aqueous buffer.

Because the disruption of the water hydration layer at the protein surface would destabilize the molecules in solution, the use of ethanol in the ERS preparation is likely to at least partially precipitate pancreatic α-amylase from solution. Since the ERS is collected by centrifugation, the addition of ethanol prior to centrifugation would be expected to increase the contamination of the ERS with the enzyme. In order to reduce
the contamination of ERS with pancreatic α-amylase, the ERS preparation procedure was modified to collect the undigested starch at the end of the 16 h pancreatic α-amylase digestion without addition of ethanol to produce ERS<sub>L-AMY</sub>.

The proportion of ERS and ERS<sub>L-AMY</sub> from HAMS was very similar (53.1± 3.1 and 51.3± 0.8, respectively), indicating that the addition of ethanol did not influence the recovery of the starch resistant to 16 hours pancreatic α-amylase digestion. However, the residual amylolytic activity associated with ERS was reduced about 10 fold in ERS<sub>L-AMY</sub> (Table 4.18), indicating that most of the pancreatic α-amylase that was collected with ERS resulted from the use of ethanol. Nonetheless, residual amylolytic activity associated with ERS<sub>L-AMY</sub> was still observed.

Binding studies have indicated the presence of a starch binding site on pancreatic α-amylase (Alkazaz et al. 1996, Qian et al. 1995). Therefore, coprecipitation of pancreatic α-amylase with the ERS<sub>L-AMY</sub> might result from specific binding of pancreatic α-amylase to ERS<sub>L-AMY</sub>. However, the coprecipitation of pancreatic α-amylase with the ERS<sub>L-AMY</sub> might also result from non-specific entrapment of the enzyme in the precipitated ERS<sub>L-AMY</sub>. The pH of the maleate buffer used in the pancreatic α-amylase digestion described by the official method for in vitro RS determination is 6.0. Because the isoelectric point of porcine pancreatic α-amylase (pI 5.95) (Desseaux et al. 1988), and the minimum solubility for a protein is at pH = pI, coprecipitation of pancreatic α-amylase with the ERS<sub>L-AMY</sub> might also result from low solubility of the enzyme in the pH 6.0 solution.

Faber et al. (2008) showed that the highest solubility of pancreatic α-amylase is in the presence of thiocyanate ions. Therefore, to reduce amylase contamination, ERS<sub>L-AMY</sub> was suspended in potassium thiocyanate (pH 7.2) with the intent to re-solubilize the pancreatic α-amylase that may have coprecipitated with ERS<sub>L-AMY</sub>. If pancreatic α-amylase re-solubilizes in the potassium thiocyanate solution, less enzyme should be associated with the starch and more would be present in solution. Starch-free supernatant (SFS) was obtained by centrifuging and filtering the ERS<sub>L-AMY</sub> in potassium thiocyanate dispersion. The amylolytic activity, as estimated by utilizing the soluble-starch agar plate assay, of the SFS should increase as a result of solubilization of the pancreatic α-amylase
in the potassium thiocyanate solution. Because the amylolytic activity of the SFS did not increase as a consequence of the potassium thiocyanate treatment (Table 4.20), any residual pancreatic α-amylase apparently could not be removed from the ERS_L-AMY by re-solubilization of the enzyme in the potassium thiocyanate solution.

Because the residual amylolytic activity of ERS_L-AMY could not be reduced by re-solubilization in potassium thiocyanate, the use of a pancreatic α-amylase inactivating agent was investigated. The activity of a bacterial α-amylase in pure solution has been shown to be irreversibly reduced by incubation in EDTA solution at 40°C (Lecker and Khan 1996, 1998). EDTA is a calcium-chelating ligand, which inactivates pancreatic alpha-amylase by dissociating the Ca^{2+} from the active enzyme molecule, causing denaturation of the enzyme.

In the present study, the residual amylolytic activity associated to ERS_L-AMY, as a function of the incubation time in the EDTA solution, was estimated by using the soluble-starch agar plate assay. In this assay, aliquots of the ERS_L-AMY treated with EDTA are obtained overtime. The starch is first re-suspended in MRS broth, and then transferred to the wells on the agar plate. The radius of the unstained zones formed on the agar plate decreased with increasing the EDTA incubation time, indicating that the residual amylolytic activity associated with ERS_L-AMY decreased overtime during the incubation in EDTA (Fig. 4.30). For the sample obtained by 24 h incubation in EDTA solution (referred to as ERS_VL-AMY), the released activity of the associated pancreatic α-amylase was reduced by 99.2% (Table 4.23). Because the MRS broth contains Ca^{2+} and suspending the ERS_L-AMY treated with EDTA in MRS did not seem to reactivate the enzyme, it can be expected that the reduction in the residual amylolytic activity associated with ERS_L-AMY resulted from irreversible denaturation of most of the pancreatic α-amylase associated to the ERS_L-AMY by EDTA.

5.8.2 Extent of released pancreatic α-amylase associated with ERS_L-AMY

The residual amylolytic activity released by ERS_L-AMY for HAMS, HMT, HAMS 2%R-20U and CCS 2%R-20U was indirectly estimated by utilizing the soluble-starch agar plate assay. As described above (section 5.6.3), it has been shown that the radius of
the zone formed on the soluble starch-containing agar plates depends directly on the level of pancreatic α-amylase placed in the wells. However, for the residual activity associated with ERS<sub>L-AMY</sub>, the outcome would be more complicated.

The full extent of amylolytic activity associated with ERS<sub>L-AMY</sub> would be underestimated by utilizing the soluble-starch plate assay for two reasons. 1) The association of pancreatic α-amylase with ERS<sub>L-AMY</sub> could result from specific binding to ERS<sub>L-AMY</sub>, or from non-specific entrapment of the enzyme in the ERS<sub>L-AMY</sub>. In both cases, the release of residual pancreatic α-amylase would occur over time. Therefore, the amount of pancreatic α-amylase that diffuses radially from the well would increase over time, as new free amylase is produced. As a result, the radius of the zones formed on the soluble starch-containing agar plates by ERS<sub>L-AMY</sub> would be smaller than if the same total amount of enzyme began to diffuse at time zero. 2) Pancreatic α-amylase could still be specifically bound to ERS<sub>L-AMY</sub> even at the end of the incubation time of the assay; the amount released would not necessarily be all that had been associated with the ERS<sub>L-AMY</sub>.

Since the soluble-starch plate assay could not be utilized to accurately estimate the total level of amylolytic activity associated with ERS<sub>L-AMY</sub>, the total amount of pancreatic α-amylase associated with ERS<sub>L-AMY</sub> from different sources is still an open question. Residual amylolytic activity released by ERS<sub>L-AMY</sub> was still observed for all four ERS<sub>L-AMY</sub> investigated (Table 4.19). To determine whether the residual pancreatic α-amylase makes an important contribution to further digestion of ERS<sub>L-AMY</sub>, the following experiment was performed.

### 5.8.3 Further digestion of ERS<sub>L-AMY</sub>

The time course of further digestion of ERS<sub>L-AMY</sub> from HAMS, HMT, CCS 2%R-20U and HAMS 2%R-20U with added pancreatic α-amylase indicated that each of the ERS<sub>L-AMY</sub> was to some extent further digested by pancreatic α-amylase (Fig. 4.29). This outcome is consistent with the finding described in section 5.5, supporting the argument that ERS is not a fully pancreatic α-amylase-resistant residue.

Enzyme buffer containing added pancreatic α-amylase (6 U/ml) was used for the digestion in the initial digestion extended to 48 hours (Fig. 4.25) and in the digestion of
ERSL-AMY recovered after 16 hours (Fig. 4.29). In the initial digestion, the enzyme buffer was added to 20 mg of starch sample. For the further digestion of ERSL-AMY, the enzyme buffer was added to the starch that resisted the 16 h pancreatic α-amylase digestion (ERSL-AMY). Because the amount of ERSL-AMY was less than the original 20 mg, the level of pancreatic α-amylase per mg of starch was greater in the further digestion of ERSL-AMY than in the initial digestion. Because the level of added pancreatic α-amylase per mg of starch for the two assays differs, and because some residual pancreatic α-amylase was present even on ERSL-AMY, the kinetics of the two assays should not be too precisely quantitatively compared. However, both assays indicated that HMT has the lowest residual susceptibility to pancreatic α-amylase digestion after 16 hours (Fig. 4.25 and 4.29).

The time course of further digestion of ERSL-AMY from HAMS, HMT, CCS 2%R-20U and HAMS 2%R-20U by the residual pancreatic α-amylase associated with ERSL-AMY was investigated by incubating the ERSL-AMY in buffer without added pancreatic α-amylase (Fig. 4.29). For ERSL-AMY from HAMS, CCS 2%R-20U and HAMS 2%R-20U, although the time to accomplish digestion was shorter in the presence of added pancreatic α-amylase, substantial digestion was accomplished even in buffer without added pancreatic α-amylase. This outcome indicated that for these samples, the residual pancreatic α-amylase makes an important contribution to further digestion of ERSL-AMY.

For ERSL-AMY from HMT, digestion was independent of the additional pancreatic α-amylase. The resistance to pancreatic α-amylase digestion of HMT has been attributed to highly ordered helical associations (Rees 2008). It appears that the limiting factor in digestion for ERSL-AMY from HMT is likely the nature of the substrate, not the activity of the enzyme. It is possible that all susceptible substrate sites may be occupied by the residual pancreatic α-amylase.

The association of pancreatic α-amylase to ERSL-AMY could result from specific binding to starch or from non-specific entrapment of the enzyme in the ERSL-AMY. Although the total amount of pancreatic α-amylase associated with ERSL-AMY from different sources could not be precisely estimated (see previous section), results indicate that the pancreatic α-amylase associated with ERSL-AMY can indeed continue the
digestion of ERS_L-AMY from HAMS, HMT, CCS 2%R-20U and HAMS 2%R-20U. The implication is that, physiologically, the pancreatic α-amylase associated with RS could pass from the small intestine to the colon together with the RS. The pancreatic α-amylase associated with RS could further digest the RS in the colon, and amylolytic activity from colonic organisms may not be essential for utilizing RS. This is a potentially important contribution to the literature, as it is presumed that RS hydrolysis occurs by the amylolytic enzymes of the bacterial microflora of the large intestine. Products of RS degradation in the colon by action of the associated pancreatic α-amylase could serve as sources of lower-molecular weight carbohydrates to be converted to fermentable substrates by the autochthonous colonic microbiota.

5.8.4 Significance of the residual amylolytic activity associated with ERS_L-AMY and ERS_VL-AMY from HAMS on the growth of selected Bifidobacterium strains

The objective of this study was to compare growth of three Bifidobacterium strains differing in their extracellular amylolytic activity (section 5.6.4) when prepared resistant starches from HAMS with different residual levels of pancreatic α-amylase (ERS_L-AMY and ERS_VL-AMY) are used as the main source of carbohydrate.

Pancreatic α-amylase associated with ERS_L-AMY was shown to be effective in continuing the ERS_L-AMY digestion (Fig. 4.29). It was hypothesized that products of RS degradation by action of the associated pancreatic α-amylase could serve as sources of fermentable substrates for Bifidobacterium strains, particularly for the strains with limited ability to degrade starch. To test this hypothesis, every effort to remove the amylolytic activity associated with ERS_L-AMY maintaining the physical characteristics of the starch substrate was made (section 5.8.1). However, the residual amylolytic activity associated with the RS obtained from HAMS could not be completely eliminated, but only reduced by 99.5% (Table 4.19) even by the most extensive treatment tested, which consisted in 24 hours incubation of ERS_L-AMY in EDTA solution to produce ERS_VL-AMY. Because of the much lower levels of residual amylolytic activity associated with ERS_VL-AMY, products of further ERS_VL-AMY digestion would be expected to serve as sources of fermentable
substrates for *Bifidobacterium* strains to a much lower extent than products of further ERS\(_{L-AMY}\) digestion.

Of the three strains of *Bifidobacterium* investigated, *B. pseudolongum* ATCC 25526 had the greatest ability to degrade starch, as defined by the agar plate assay (Table 4.15). Nevertheless, when ERS\(_{L-AMY}\) or ERS\(_{VL-AMY}\) were used as the main carbohydrate source growth (defined as increase of viable cells count overtime) of *B. pseudolongum* ATCC 25526 over the first 24 hours was similar to the no-carbohydrate-added MRS broth (no-CHO-added control) (Fig. 4.31). This outcome suggests that *B. pseudolongum* ATCC 25526 did not utilize ERS\(_{VL-AMY}\), ERS\(_{L-AMY}\) or products of the continued digestion by the pancreatic α-amylase associated with ERS\(_{L-AMY}\) and ERS\(_{VL-AMY}\) as fermentable substrates for growth.

For *B. animalis* subsp. *lactis* DSMZ 10140, cell counts by 24h were ~1 log CFU/ml higher when ERS\(_{L-AMY}\) was used as the main carbohydrate source, as compared to the no-CHO-added control. Somewhat higher counts than the no-CHO-added control were observed also for ERS\(_{VL-AMY}\). ERS\(_{L-AMY}\) and ERS\(_{VL-AMY}\) would be mainly residual HAMS granules that were hollow on the inside (Rees 2008). Therefore, ERS\(_{L-AMY}\) and ERS\(_{VL-AMY}\) must be converted to lower-molecular weight carbohydrates prior being internalized and fermented by a microorganism. The conversion of ERS\(_{L-AMY}\) and ERS\(_{VL-AMY}\) to lower-molecular weight carbohydrates could result from either the action of 1) extracellular amylolytic enzymes, but no indication of synthesis of extracellular amylolytic activity was observed for *B. animalis* subsp. *lactis* DSMZ 10140 by the agar plate assay (Table 4.15); 2) membrane-associated enzymes. The agar plate assay would not allow membrane-associated hydrolysis to be evaluated. However, starch degradation by the action of membrane-associated enzymes is usually associated with adhering of the bacterial cell to the starch surface (Anderson and Salyers 1989a, b, van den Broek et al. 2008). Because there is no evidence for adhesion of *B. animalis* subsp. *lactis* DSMZ 10140 to starch (Chittiprolu 2009), ERS\(_{L-AMY}\) and ERS\(_{VL-AMY}\) conversion to lower-molecular weight carbohydrates by membrane-associated enzymes seems unlikely for this microorganism. The observed growth may be more likely due to fermentation of products of ERS\(_{L-AMY}\) and ERS\(_{VL-AMY}\) degradation by action of the associated pancreatic α-amylase.
Reduction of viable cells count overtime should be understood as a reduced number of surviving cells. It appears that for *B. animalis* subsp. *lactis* DSMZ 10140, both ERS<sub>L-AMY</sub> and ERS<sub>VL-AMY</sub> enhanced survival after 24h as compared to the no-CHO-added control. It can be assumed that the reduction in the number of surviving cells likely results from slower exhaustion of the carbohydrate source in the medium. Thus, enhancement of cells survival would result from a greater presence of a source of fermentable carbohydrate in the media. Both the products of continued ERS<sub>L-AMY</sub> and ERS<sub>VL-AMY</sub> pancreatic α-amylase digestion seem to provide sufficient fermentable substrate to enhance survival of *B. animalis* subsp. *lactis* DSMZ 10140 as compared to the no-CHO-added control.

A different growth trend was observed for *B. animalis* subsp. *lactis* BB12 (Fig. 4.31). By 24h, the strain grew better when ERS<sub>L-AMY</sub>, as compared to ERS<sub>VL-AMY</sub>, was used as the main carbohydrate source. However, by 48h, the strain kept growing when ERS<sub>VL-AMY</sub> was used as the main carbohydrate source, while the number of surviving cells decreased when ERS<sub>L-AMY</sub> was used as the main carbohydrate source. As for the previous organism, no evidence for adhesion of *B. animalis* subsp. *lactis* BB12 to starch was found in previous work (Chittiprolu 2009). Although the ability of *B. animalis* subsp. *lactis* BB12 to degrade starch extracellularly, as defined by agar plate assay, is somewhat limited (Table 4.15), it appears that *B. animalis* subsp. *lactis* BB12 has the ability to at least partially degrade ERS<sub>VL-AMY</sub>, and utilize the products of ERS<sub>VL-AMY</sub> degradation as main carbohydrate source.

Because *B. animalis* subsp. *lactis* BB12 grew better in a shorter time when ERS<sub>L-AMY</sub>, as compared to ERS<sub>VL-AMY</sub>, was used as the main carbohydrate source, it is likely that the strain utilized a higher level of the products of further digestion by the pancreatic α-amylase associated with ERS<sub>L-AMY</sub> as fermentable substrates. It is possible that lower availability of the carbohydrate sources (or carbon sources) in the media containing the ERS<sub>VL-AMY</sub> induced a greater production of extracellular amylolytic enzymes. These enzymes would continue to degrade ERS<sub>VL-AMY</sub> overtime, providing more lower-molecular weight carbohydrates than the one that could be provided by degradation of ERS<sub>L-AMY</sub>. The greater availability of lower-molecular weight carbohydrates would possibly enhance cells survival overtime, as seen for ERS<sub>VL-AMY</sub>. 

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5.9 Growth of selected strains of Bifidobacterium on ERS\textsubscript{VL-AMY} from four sources

A main question of the present thesis project was whether RS from different sources may lead to differences in RS utilization and fermentability by a particular strain of \textit{Bifidobacterium} that had extracellular amylolytic activity capable of degrading RS-containing starch. In order to investigate this question, putative RS had to be 1) obtained from the RS-containing starch materials. Undigested starch that should be reasonably representative of RS \textit{in vivo} was obtained by using the official \textit{in vitro} determination of RS procedure; 2) added to the media by maintaining the integrity of the RS physical structure. During the preparation procedure to obtain the RS, the presence of residual amylolytic activity associated with the recovered RS was observed. To decrease a possibly artefactual level of associated pancreatic \(\alpha\)-amylase, the putative RS was recovered without ethanol precipitation. Nonetheless, residual pancreatic \(\alpha\)-amylase was observed even for the RS recovered without ethanol precipitation. Because the residual pancreatic \(\alpha\)-amylase was capable of further hydrolyzing the RS, every effort was made to reduce the amylolytic activity associated with the recovered RS without losing integrity of the RS structure. Although a 24h EDTA treatment was used to reduce the residual amylolytic activity of the recovered RS by more than 95\% (ERS\textsubscript{VL-AMY}), the total level of residual pancreatic \(\alpha\)-amylase associated with the ERS\textsubscript{VL-AMY} could not be quantified. Nonetheless, because the residual amylolytic activity was heavily reduced by the EDTA treatment, it was assumed that substantially greater bacterial growth relative to a no-CHO-added control would be observed when the recovered RS was used as the main source of carbohydrate only if the \textit{Bifidobacterium} strain tested had the ability to utilize the RS.

ERS\textsubscript{VL-AMY} from HAMS, HMT, CCS 2\%R-20U and HAMS 2\%R-20U were used as main carbohydrate source for the growth of three bifidobacterial strains. \textit{B. pseudolongum} ATCC 25526 counts were higher by 24 h on all ERS\textsubscript{VL-AMY} analyzed than the no-CHO-added control (Fig. 4.32). This outcome suggested that greater growth could be due to the extracellular amylolytic enzymes synthesized by \textit{B. pseudolongum} ATCC
25526 degrading at least some of the ERS<sub>VL-AMY</sub> for each type of ERS<sub>VL-AMY</sub> analyzed. The products of ERS<sub>VL-AMY</sub> degradation could then be used by the bacterial strain as the main source of carbohydrate for growth.

By 72 h, survival of <i>B. pseudolongum</i> ATCC 25526 greatly differed depending on the ERS<sub>VL-AMY</sub> source. The survival rate was the greatest when the strain was grown on ERS<sub>VL-AMY</sub> from CCS 2%R-20U or HAMS 2%R-20U, and the lowest when grown on ERS<sub>VL-AMY</sub> from HMT. The undigested starch recovered from HAMS, HMT, CCS 2%R-20U and HAMS 2%R-20U by using the official <i>in vitro</i> determination of RS is not a fully pancreatic α-amylase-resistant residue (section 5.8.3). Further digestion of the recovered RS showed that the recovered RS from HMT had the lowest residual susceptibility to pancreatic α-amylase digestion among the four samples tested. While the limiting factor in further pancreatic α-amylase digestion of RS from HAMS, CCS 2%R-20U and HAMS 2%R-20U is the kinetics of digestion; the limiting factor in further pancreatic α-amylase digestion of RS from HMT is the refractory nature of the substrate. Physical access of pancreatic α-amylase to the chains of the RS from HMT may be limited by a great extent of double-helical associations and alignment (Rees 2008). These very same double helices could limit the physical access of the bacterial extracellular amylolytic enzymes to the chains of the RS from HMT. It is likely that the extracellular amylolytic enzymes of <i>B. pseudolongum</i> ATCC 25526 could initially degrade some of the ERS<sub>VL-AMY</sub> from HMT and utilize the products of degradation for the observed growth by 24h. However, degradation of the ERS<sub>VL-AMY</sub> from HMT beyond 24h was so limited that even the survival of the strain was compromised.

For <i>B. choerinum</i> ATCC 27686, substantial growth through 12 h was observed on all ERS<sub>VL-AMY</sub> analyzed relative to the no-CHO-added control (Fig. 4.32). Growth continued through 24 h on ERS<sub>VL-AMY</sub> from CCS 2%R-20U and HAMS 2%R-20U; while cells survival rapidly decreased by 24 h on ERS<sub>VL-AMY</sub> from HMT. It appears that, although <i>B. choerinum</i> ATCC 27686 could degrade at least some of all the ERS<sub>VL-AMY</sub> for each type of ERS<sub>VL-AMY</sub> analyzed, the growth of this strain was most favored on the starch substrates with greatest residual pancreatic α-amylase susceptibility.

The growth of <i>B. infantis</i> ATCC 15697 on each type of ERS<sub>VL-AMY</sub> analyzed was similar to the no-CHO-added control (Fig. 4.32). For this strain, although extracellular
amylolytic activity was observed by the agar plate assay, there was no evidence that any ERS_{VL-AMY} was utilized as main carbohydrate source for growth. Nonetheless, the presence of ERS_{VL-AMY} from HAMS, CCS 2\%R-20U and HAMS 2\%R-20U favored cell survival over the no-CHO-added control and the ERS_{VL-AMY} from HMT. Because substantial growth was observed on the no-CHO-added control, it may be suggested that other components in the medium, e.g. proteins, may be the preferred energy source rather than carbohydrates. The greater survival rate of may indicate that fermentation of the products of ERS_{VL-AMY} degradation by the bacterial extracellular amylolytic enzymes may be utilized by this strain upon exhaustion of the preferred energy source.

Evidence for adhesion of B. pseudolongum ATCC 25526 (Chittiprolu 2009, Crittenden 2001) B. choerinum ATCC 27686 (Chittiprolu 2009) to HAMS, but not for B. infantis ATCC 15697 (Chittiprolu 2009), has been shown in previous work. Although the adhesion degree has been show to varies according to the starch substrate (Chittiprolu 2009), it is likely that the adhesion of B. pseudolongum ATCC 25526 and B. choerinum ATCC 27686 cells to the ERS_{VL-AMY} could result in artefactual lower counts by the methodology used in this study. Examination of the level of adhering cells to the ERS_{VL-AMY} by microscopy, as in Chittiprolu (2009) and O’Riordan et al. (2001), would have helped accounting for the artefactual lower counts.

ERS_{VL-AMY} from HAMS was used as main source of carbohydrate for growth of B. pseudolongum ATCC 25526 in both the current experiment and in the experiment described in the previous section. In the previous experiment, at 24 h there was no evidence for greater viable cells counts than for the no-CHO-added control (Fig. 4.31). Within each experiment, each strain was grown either on ERS_{VL-AMY} from HAMS or on the no-CHO-added control in triplicate starting on the same day. This experimental design allows for comparison of utilization of putative RS’s within each strain. It does not allow for comparison of putative RS’s utilization between strains, nor can it evaluate growth differences on different days. The lack of agreement in the viable cells counts for B. pseudolongum ATCC 25526 in the two experiments calls in to attention for possible effects of day-to-day variation in bacterial growth on putative RS utilization.

The present study is the first study to address the question whether a particular bifidobacterium strain can hydrolyze putative RS in vitro. This research shows that
*Bifidobacterium* strains can differently utilize different forms of putative RS *in vitro*. Although it would be inappropriate to conclude about differences in the extent of colonic degradation of the four starches *in vivo*, as other amylolytic activities in the colon are likely to act on each RS, this study represents an important advance in the understanding of RS as substrate for *Bifidobacterium* strains.
Chapter 6 : Conclusion

The goal of this project was to produce and characterize RS-containing starch materials with different susceptibilities to pancreatic \( \alpha \)-amylase, and compare the growth of selected strains of \textit{Bifidobacterium} when prepared resistant starches with different susceptibility to continued pancreatic \( \alpha \)-amylase digestion are used as the main source of carbohydrate.

RS-containing starch preparations were obtained by rehydration (1% or 2% starch concentration) in the presence of variable levels of isoamylase (0, 10 or 20U/g of starch) of an ethanol precipitate of common corn starch (CCS) or high-amyllose maize starch (HAMS) that had been molecularly dispersed in 0.5N NaOH. Regardless of the starch concentration at rehydration and debranching by isoamylase, recovery of starch molecules from aqueous dispersion was greater for the HAMS than the CCS samples, indicating that dispersed HAMS has a greater tendency toward molecular aggregation than dispersed CCS.

For both CCS and HAMS samples, rehydration in the absence of isoamylase of the ethanol precipitates led to proportional coprecipitation of amylose and amylopectin from dispersion. DSC evidence suggests that amylopectin inhibited the ability of amylose to form long double helices. X-ray diffraction data shows that whatever amylose-amylose double helices and mixed double helices between amylose and amylopectin existed in the rehydrated ethanol-precipitated CCS and HAMS did not contribute to crystallinity in the samples. The non-crystalline double helices were not associated with resistance to enzymatic digestion for the CCS samples, but they did for the HAMS samples. The longer non-crystalline double helices in the HAMS samples than in the CCS samples could account for the difference in RS content between the samples.

For CCS, greater recovery of starch after rehydration in an aqueous dispersion resulted from debranching by isoamylase during rehydration; while for HAMS, the
recovery of starch from aqueous dispersion occurred independent of debranching by isoamylase. For both CCS and HAMS, debranching during rehydration led to preferential co-precipitation of amylose and the longer linear chains generated from amylpectin debranching. The co-precipitation of amylose with the linear chains was mainly in form of mixed amylose-linear chain double helices. Debranching of starch molecules during rehydration was effective in generating RS even for CCS, a starch that has essentially no RS when the ethanol-precipitated CCS is simply rehydrated. Debranching by isoamylase favored the alignment of the starch double helices, leading to development of a somewhat increased crystallinity in the precipitates. Although little increase in crystallinity resulted from HAMS so treated, for CCS the RS content increased with increasing of crystallinity.

Because of the high RS content, the RS-containing starch preparations prepared by rehydration at 2% starch concentration in the presence of 20U isoamylase of the ethanol-precipitated CCS (CCS 2%R-20U) and HAMS (HAMS 2%R-20U) were selected for further characterization. For these samples, enzyme resistance was attributed to a combination of type 1 and type 3 RS. The proportion of type 1 RS was found to decrease as particle size decreased, and it did not influence the chemical nature of the RS recovered. The RS content following thermal treatment of the CCS 2%R-20U and HAMS 2%R-20U samples was independent of particle size. A greater proportion of longer double helices in thermally treated RS than in RS was attributed to the melting of the shorter chains during the thermal treatment and to the double helical association of the then mobile longer chains during the thermal treatment or during cooling.

The time course of digestion for the selected prepared RS-containing starch preparations (CCS 2%R-20U and HAMS 2%R-20U) was compared to the time course of digestion of two commercial RS-containing starch materials (HAMS and HMT). Because a limit for pancreatic α-amylase digestion was not reached within the stipulated 16 h digestion of the official method for in vitro RS determination, RS is not a fully pancreatic α-amylase-resistant residue. Kinetic analysis of the digestion time course showed that the RS from HMT had the lowest residual susceptibility to pancreatic α-amylase digestion among the four samples tested. While the limiting factor in further pancreatic α-amylase digestion of RS from HAMS, CCS 2%R-20U and HAMS 2%R-20U was the kinetics of digestion; the limiting factor in further pancreatic α-amylase digestion of RS from HMT
was the refracting nature of the substrate. Physical access of pancreatic \( \alpha \)-amylase to the chains of the RS from HMT may be limited by a great extent of helical associations and helical alignment. Since the 16 h digestion of the official method for \textit{in vitro} determination of RS has been validated against \textit{in vivo} resistance in human subjects, RS’s with greater residual susceptibility to pancreatic \( \alpha \)-amylase, such as the RS from HAMS, CCS 2\%R-20U and HAMS 2\%R-20U, might be more susceptible to hydrolysis in the colon. This increased susceptibility would thus favor hydrolysis in the proximal colon.

Evidence for the presence of residual pancreatic \( \alpha \)-amylase associated with the recovered RS from the four samples analyzed was found. Although the methodology used in the present study could not accurately quantify the total level of residual pancreatic \( \alpha \)-amylase associated with the RS, the continued digestion of the RS by the action of the residual pancreatic \( \alpha \)-amylase was substantial. The association of pancreatic \( \alpha \)-amylase with the RS did not seem to be an artifact of the RS preparation procedure, as the coprecipitation of pancreatic \( \alpha \)-amylase with the starch was not due to low stability of the enzyme in solution. The association of the pancreatic \( \alpha \)-amylase with the RS could result from specific binding and/or non-specific physical entrapment of the enzyme in the starch samples. The presence of pancreatic \( \alpha \)-amylase in putative RS has not been reported in the literature. The physiological implication is that pancreatic \( \alpha \)-amylase could move to the colon together with the RS, and contribute to further digest the RS in the colon. The products of the further RS digestion could serve as fermentable carbohydrates for the colonic microbiota.

An assay to investigate the ability of strains of \textit{Bifidobacterium} to degrade starch was developed based on the methods of Wang et al. (1999) and Ryan et al. (2006). The methods currently present in the literature were modified so that the integrity of the original physical structure of the starch materials tested could be maintained. The newly developed assay could then be used to investigate whether the native granular structure of HAMS may lead to differences in RS microbial utilization by strains of \textit{Bifidobacterium}.

Three strains of \textit{Bifidobacterium}, selected for their different ability to extracellularly degrade granular HAMS, were used to investigate whether further digestion of putative RS by the action of the residual pancreatic \( \alpha \)-amylase might
influence bacterial growth and survival. Further RS digestion by residual pancreatic α-amylase had no effect on the growth or survival of the bifidobacterial strain with the greatest ability to extracellularly degrade starch (*B. pseudolongum* ATCC 25526); while it enhanced growth and survival of the strain with the lowest ability to extracellularly degrade starch (*B. animalis* subsp. *lactis* DSMZ 10140). The third strain (*B. animalis* subsp. *lactis* BB12) grew better over a shorter time but had lower cells survival in the presence of the products of further RS digestion. The significance of the residual amylolytic activity associated with putative RS on the growth of *Bifidobacterium* strains has not been investigated in the literature. Results from the present study indicate that further RS digestion due to associated pancreatic α-amylase could differentially benefit the numerous strains of bifidobacterium in the colon.

Finally, the growth of three strains of *Bifidobacterium* with great ability to degrade HAMS extracellularly (*B. pseudolongum* ATCC 25526, *B. choerinum* ATCC 27686 and *B. infantis* ATCC 15697) was investigated when the RS from HAMS, HMT, CCS 2%R-20U and HAMS 2%R-20U were used as the main source of carbohydrate, after making every effort to remove associated amylolytic activity. For all three strains, the putative RS with lowest residual susceptibility to pancreatic α-amylase digestion, RS from HMT, had a less favorable effect on viable cell counts. Further susceptibility to pancreatic α-amylase digestion of the RS from HMT is limited by a great extent of double-helical associations and double-helical alignment. Similarly, these double-helical structures could limit the susceptibility of RS from HMT to the bacterial extracellular amylolytic hydrolysis. Thus, the susceptibility of RS to further amylolysis may influence the ability of specific *Bifidobacterium* strains to utilize the RS for growth *in vitro*.

The present study is the first study to address the question whether a particular bifidobacterium strain can hydrolyze putative RS *in vitro*. This research shows that *Bifidobacterium* strains can differently utilize different forms of putative RS *in vitro*. It would be inappropriate to conclude about differences in the extent of colonic degradation of the four starches *in vivo*, as other amylolytic activities in the colon are likely to act on each RS. Whether a greater residual susceptibility to hydrolysis would be of benefit to bifidobacterium species in the colon in the presence of an intact complex microbiota is not clear from this work. It does seem likely that hydrolysis would be favored in proximal
regions of the colon. Fermentation of the products of RS hydrolysis in the distal colonic regions might be more advantageous from a health standpoint, since those are the regions of the gut where most cancers take place.
Chapter 7 : Suggestions for future work

7.1 Effect of amylose and amylopectin structure on starch recovery and digestibility

The greater tendency to molecular aggregation as well as the lower susceptibility to digestion observed for the HAMS as compared to CCS may be attributable to the higher amylose content, to the chain length distribution of amylopectin and to the molecular size of amylose and amylopectin. The CCS used in this study is about 27% amylose, while the HAMS is about 73% amylose. It would be interesting to generate RS-containing starch materials from CCS fractions with amylose/amylopectin proportion of 75/25 and from HAMS fractions with amylose/amylopectin proportion of 25/75.

7.2 Hydrothermal treatment

One of the main ideas behind this project was that manipulation of starch structure can be used to produce materials with different RS values. In order to obtain the new structures, CCS and HAMS were first molecularly dispersed in sodium hydroxide to eliminate any physical structure present in the starting materials. To further enhance the RS values and the TT-RS values, the prepared precipitates could be subjected to hydrothermal treatments. Polymers are routinely annealed by heat treatment to temperature below the melting temperature. Because the melting endotherms for the RS-containing starch materials were very broad, a number of different temperatures could be chosen for annealing. The effects of the annealing treatment on the residual susceptibility to pancreatic $\alpha$-amylase of the RS could also be investigated.

7.3 Physical structure of recovered RS

For the RS-containing starch materials, resistance to enzymatic digestion was attributed to both crystalline and non-crystalline double helices. It would be interesting to examine the physical properties by WAXD, NMR and DSC of the recovered RS. It might
be possible to gain insight into pattern of enzymatic hydrolysis with respect to the RS-containing starch materials, and to relate the residual susceptibility to further amylolysis of the recovered RS to physical structure.

7.4 Investigation of the residual pancreatic α-amylase associated with RS

The methodology used in the present study could not quantify the total level of residual pancreatic α-amylase associated with the putative RS samples, only the levels of pancreatic α-amylase that were released under various conditions. Thus, it is unclear how much residual pancreatic α-amylase remained associated with the putative RS. Moreover, methodology used in the present study could not be used to indicate whether the association of the pancreatic α-amylase with the putative RS would result from specific binding or non-specific physical entrapment of the enzyme in the starch samples. The use of biochemical assays, like SDS gel electrophoresis, could be helpful in gathering insight in the level and type of association of residual pancreatic α-amylase associated with the putative RS samples.

7.5 Determination of the extracellular amylolytic enzymes synthesized by bifidobacterial strains involved in starch degradation

Most of the Bifidobacterium strains investigated in the present study showed the ability to at least partially degrade soluble-starch. The putative extracellular amylolytic enzymes involved in starch degradation for bifidobacterium strains are still unknown. It would be interesting to identify the extracellular amylolytic enzymes for at least the strains of Bifidobacterium that showed the greatest ability to degrade starch. The cell/starch-free supernatant containing the extracellular amylolytic enzymes could be obtained by following the protocol described in Wang et al. (1999). A possible strategy to identify these enzymes may consist in using 2D electrophoresis; for example, by coupling to the use of zymogram with subsequent isoelectric focusing electrophoresis. By using a zymogram, the proteins in the cell/starch-free supernatant would be separated by mass, using a polyacrylamide gel. The bands where amylolytic activity is observed could be collected and analyzed by isoelectric focusing. By isoelectric focusing, the native
proteins present in the band collected from the zymogram gel would be separated by their isoelectric point. By using the mass, the type of amylolytic activity, and the isoelectric point, the amylolytic enzyme may be identified.

7.6 Reproducibility of growth studies using putative RS as main carbohydrate source for bifidobacterium strains

A lack of agreement in the viable cells counts for *B. pseudolongum* ATCC 25526 grown on ERS_{VL-AMY} from HAMS in the experiments discussed in sections 5.8.3 and 5.9 calls in to attention for possible effects of day-to-day variation in bacterial growth on putative RS utilization. To allow for comparisons of growth differences on different days, within each experiment, each strain should be grown either on or on the no-CHO-added control in triplicate starting on three different days. The pH of the growing media should be monitored overtime. Also, the level of cells adhering to the starch substrate should be monitored by microscopy, as in Chittiprolu (2009) and O’Riordan et al. (2001), to help accounting for possible artefactual lower counts.

7.7 Physical morphology of partially degraded granular HAMS by the action by the action of extracellular amylolytic enzymes from bifidobacterium strains

The light zones formed by inoculation of selected bifidobacterium strains on the HAMS-containing agar plates were attributed to partial degradation of the HAMS granules by the bacterial extracellular enzymes during the incubation time. The partially degraded HAMS granules could be obtained from the light zones formed on the agar plate. The physical morphology of these partially degraded HAMS granules could be analyzed using light microscopy and scanning electron microscopy (SEM).
References


Chittiprolu, S. 2009. Effect of starch spherulites on survival of bifidobacteria in presence of acid or bile in: Food Science. The Pennsylvania State University, University Park.


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Appendix A:

Determination of the proportion of crystallinity
Figure A1 Illustration of a) the total area above the baseline; and b) the area of the crystalline portion used to calculate the proportion of crystallinity for the preparation obtained by rehydration of the CCS ethanol precipitate at 1% starch concentration, in the presence of 20U isoamylase (CCS 1%R-20U).
Figure A2 Illustration of a) the total area above the baseline; and b) the area of the crystalline portion used to calculate the proportion of crystallinity for the preparation obtained by rehydration of the CCS ethanol precipitate at 2% starch concentration, in the presence of 20U isoamylase (CCS 2%R-20U).
Figure A3 Illustration of a) the total area above the baseline; and b) the area of the crystalline portion used to calculate the proportion of crystallinity for the preparation obtained by rehydration of the HAMS ethanol precipitate at 2% starch concentration, in the presence of 20U isoamylase (HAMS 2%R-20U).
Appendix B:

Time course of digestion
Figure B1 Time course of digestion of the extended resistant starch assay for CCS 2%R-20U and HAMS 2%R-20U. Curves were obtained using a 5-parameter, double-exponential equation. Curves shown are best fits of analysis of pooled data from 3 independent digestions.
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