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Effect of Gelatinization and Hydrolysis Conditions on the Selectivity of Starch Hydrolysis with α-Amylase from *Bacillus licheniformis*

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Enzymatic hydrolysis of starch can be used to obtain various valuable hydrolyzates with different compositions. The effects of starch pretreatment, enzyme addition point, and hydrolysis conditions on the hydrolyzate composition and reaction rate during wheat starch hydrolysis with α-amylase from *Bacillus licheniformis* were compared. Suspensions of native starch or starch gelatinized at different conditions either with or without enzyme were hydrolyzed. During hydrolysis, the oligosaccharide concentration, the dextrose equivalent, and the enzyme activity were determined. We found that the hydrolyzate composition was affected by the type of starch pretreatment and the enzyme addition point but that it was just minimally affected by the pressure applied during hydrolysis, as long as gelatinization was complete. The differences between hydrolysis of thermally gelatinized, high-pressure gelatinized, and native starch were explained by considering the granule structure and the specific surface area of the granules. These results show that the hydrolyzate composition can be influenced by choosing different process sequences and conditions.

KEYWORDS: Native wheat starch; gelatinization; hydrolysis; α-amylase; high pressure; high temperature

INTRODUCTION

Starch can be hydrolyzed enzymatically to yield several commercially relevant hydrolyzates (*1, 2*). The enzymatic hydrolysis of starch consists of three steps: gelatinization, liquefaction, and saccharification. Gelatinization of starch is required to increase the accessibility of the substrate and to enhance the hydrolysis rate. During liquefaction, the viscosity of the reaction mixture is reduced and gelatinized starch is partially hydrolyzed to form a product with a dextrose equivalent that varies between 15 and 30. During saccharification, these partially hydrolyzed starch chains are broken down into glucose, maltose, maltotriose, and some higher oligomers. The dextrose equivalent varies between 40 and 98 depending upon the enzyme used.

The degree of gelatinization is an important parameter during the enzymatic hydrolysis of starch. The degree of gelatinization is affected by temperature, pressure, starch concentration, and treatment time (*3–6*). For additional information on the various theories concerning thermal gelatinization, we refer to the papers of Jenkins and Donald (*7*) and Waigh et al. (*8*). Besides the degree of gelatinization, the activity and stability of the enzyme are also very important. α-Amylase (1,4-α-D-glucanohydrolase, EC 3.2.1.1) is often used for the enzymatic hydrolysis of starch. The activity and stability of α-amylase are affected by temperature, pressure, pH, substrate concentration, and additives (*9–12*).

Although the individual behavior of starch and α-amylase in aqueous solutions has been studied over a broad range of pressures and temperatures, the behavior of a system that consists of both starch and α-amylase has received less attention. The relevance of such a combined system emerges when the relation between starch gelatinization and enzymatic hydrolysis is investigated. The effects of high-pressure gelatinization and high-temperature gelatinization on the glucose production rate, the hydrolysis yield, and the enzyme activity during enzymatic hydrolysis at atmospheric pressure have been investigated before (*13–16*). However, the effects of these gelatinization conditions on the hydrolyzate composition were not determined.

The hydrolyzate composition can also be affected by the hydrolysis pressure conditions as reported by Matsunato et al. (*17, 18*). They studied the hydrolysis of various oligosaccharides by porcine pancreatic α-amylase by following the carbohydrate concentration in time at 200–400 MPa. Raabe and Knorr (*9*) hydrolyzed starch with α-amylase from *Bacillus amolyliquefaciens* using the same pressure range, but they only measured the maltose concentration in time. The effect of an

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increased pressure on the hydrolyzate composition during enzymatic starch hydrolysis with α-amylase from *Bacillus licheniformis* has not been determined before.

In the papers mentioned above, the raw materials required for enzymatic hydrolysis (starch, water, and enzymes) could be added anywhere during the process, because individually isolated components were used. It might be easier to mix all of them before gelatinization, because mixing of the enzyme with starch and water after gelatinization can be omitted. In some other cases, the raw materials required for enzymatic hydrolysis are only available as a mixture, for example, in mashing during brewing. It is therefore also relevant to determine whether the presence of the enzyme during gelatinization affects enzymatic starch hydrolysis.

The aim of this paper was to investigate the effect of starch pretreatment, enzyme addition point, and hydrolysis conditions on the hydrolyzate composition and reaction rate during enzymatic starch hydrolysis with α-amylase from *B. licheniformis*. For this purpose, the concentration of oligosaccharides (with a degree of polymerization smaller than eight), the dextrose equivalent, and the residual enzyme activity were determined as a function of time. These results can be used to determine the process configuration that should be chosen to obtain a certain hydrolyzate composition.

EXPERIMENTAL PROCEDURES

Materials. Wheat starch (S5127) was obtained from Sigma-Aldrich (Steinheim, Germany). The moisture content was 9.95 ± 0.43 w/w % (on the basis of 22 measurements and a 95% confidence interval). The moisture content was determined by drying the wheat starch in a FED 53 hot-air oven from WTB Binder (Tuttlingen, Germany) at 105 °C or in a Heraeus vacuum oven (Hanau, Germany) at 80 °C until the mass of the samples was constant in time. The water content of wheat starch was taken into account during all experiments. Thermostable α-amylase from *B. licheniformis* (Termamyl 120, 120 L, type L, activity 120 KNU/g, 1 KNU is the amount of enzyme that dextrinizes 5.26 g of starch dry substance/h at 37 °C and pH 5.6 with 0.0003 M calcium) was donated by Novozymes (Bagsværd, Denmark). The enzyme concentration used during the experiments is expressed in mass percent of this enzyme stock solution per equivalent mass of substrate (w/w %). Maltose monohydrate, fuming hydrochloric acid, sodium hydroxide, sodium chloride, calcium chloride dihydrate, calcium chloride, and trisodium phosphate were bought from Merck (Darmstadt, Germany). Maleic acid (disodium salt) was obtained from Acros Organics (Geel, Belgium). Glucose was obtained from Sigma-Aldrich (Steinheim, Germany). Milli-Q water was used for all experiments and measurements.

Amylase HR reagent (Megazyme International Ireland, Bray, Ireland) was used to measure the α-amylase activity. This reagent contains blocked (4,6-*O*-benzilidine)-*p*-nitrophenyl maltoheptaoside and excess quantities of a thermostable α-glucosidase. During the assay, endo-α-amylase cleaves a bond somewhere in this oligosaccharide. Because of the excess quantities of α-glucosidase present in the mixture, the remaining *p*-nitrophenyl maltosaccharide is quickly hydrolyzed to glucose and free *p*-nitrophenol. The amount of *p*-nitrophenol released can be measured with a spectrophotometer, and it is a measure for the α-amylase activity. The standard buffer used for all enzyme activity measurements was 0.1 M maleic acid buffer (pH 6.5) with 2 mM $CaCl_2 \cdot 2H_2O$ and 0.1 M NaCl. A solution of 0.06 M trisodium phosphate (pH 11) was used as a stopping reagent.

Microcon YM-30 centrifuge filters (Millipore Corporation, Bedford, MA) were used to remove the enzyme from the hydrolyzate. Before the actual filtration, these filters were washed by centrifugation with 500 μL of milli-Q water during 40 min at 25 °C and 13000*g*.

Methods. *Experimental Setup.* For all experiments, 5 w/w % wheat starch−water mixtures were used, because the gelatinization behavior at this starch concentration is well-investigated (*3–6*). In addition, 5 w/w % wheat starch−water mixtures can be handled easily because of the low viscosity of these mixtures. Calcium chloride (1.8 mM) was added to each reaction mixture.

High-pressure experiments (450 MPa) were carried out in a multi-vessel high-pressure apparatus (Resato FPU 100-50, Resato International B.V., Roden, The Netherlands). During these experiments, the pressure buildup rate was equal to 2.5 MPa/s, leading to a maximum temperature overshoot of 10 °C. Glycol was used as a pressure medium. Reaction mixtures were transferred to custom-made polyethylene bags (Seward Medical, London, U.K.) that were sealed with a minimum amount of air inside. Two bags were placed in each high-pressure vessel. To gelatinize starch completely, starch−water mixtures were held at 450 MPa and 50 °C for 15 min (referred to as HP treatment). The temperature, pressure, and time were slightly higher than the minimum values required for complete gelatinization to ensure that complete gelatinization was reached (*3, 6*).

For gelatinization at 0.1 MPa, a temperature-controlled batch reactor (200 mL liquid volume and 73 mm internal diameter) was used, equipped with an anchor stirrer (52 mm diameter with a stirrer speed of 300 rpm). First, wheat starch and water were mixed at room temperature in this reactor. In case the enzyme was present during gelatinization, the enzyme was also added at this point. Second, the reaction mixture was heated to 90 °C in 30 min. Subsequently, the reaction mixture was held at 90 °C for 60 min. A temperature of 90 °C is known to be sufficient to gelatinize starch completely in a 5 w/w % wheat starch−water mixture after holding it for 60 min (*3, 19, 20*). Finally, the contents of the reaction vessels were cooled down to 50 °C in 60 min. The complete heating and cooling treatment used for thermal gelatinization is referred to as the HT treatment.

In case enzyme was not present during gelatinization or gelatinization was not carried out, the enzyme was added when the temperature of the reaction mixture was 50 °C. During all experiments, the starting time of the experiment ($t = 0$) was the point at which the enzyme was added. Immediately after this point in time (in case enzyme was not present during gelatinization) or after the hydrolysis temperature was reached (in case the enzyme was present during gelatinization), 1.5 mL safe-lock tubes (Eppendorf AG, Hamburg, Germany) were filled with the reaction mixture and placed in a water bath to keep the contents of the safe-lock tubes at 50 °C. Because the reaction mixtures in the high-pressure vessels could not be stirred, the reaction mixtures that were treated at 0.1 MPa were also not stirred.

In case high-pressure gelatinized starch was used for hydrolysis at atmospheric pressure or thermally gelatinized starch was used for hydrolysis at high pressure, the gelatinized starch suspension had to be transported. During the 15 min required for transport, the temperature of the suspension was kept at 50 °C by using a thermos flask filled with water. After transportation, enzyme was mixed with the gelatinized starch suspension and, subsequently, safe-lock tubes or sample bags were filled with this mixture and placed in the water bath or high-pressure equipment for hydrolysis. The remainder of the procedure is equal to the procedures described above.

A hydrolysis temperature of 50 °C was used, because it is below the gelatinization onset temperature at atmospheric pressure (*3, 19, 20*). In this case, gelatinization would not take place during hydrolysis at atmospheric pressure and the effect of incomplete gelatinization could be investigated.

The hydrolyzate composition and enzyme activity were determined for each measurement point in time, and for that reason, two safe-lock tubes or two sample bags were used. At each sample point, sample safe-lock tubes and bags were submerged in liquid nitrogen to stop the enzyme reaction. After holding them in liquid nitrogen for approximately 15 min, the safe-lock tubes or bags were stored in a −80 °C freezer until further use.

Sample Handling. Samples from the −80 °C freezer that were taken to determine the carbohydrate composition or enzyme activity were submerged in liquid nitrogen for several minutes. Subsequently, the sample was transferred to a mortar, liquid nitrogen was added, and the sample was grinded in a mortar with a pestle.

Measurement of the Carbohydrate Composition. For measurement of the carbohydrate composition, approximately 0.3 g of the grinded sample was mixed with 1125 μL of milli-Q water and 75 μL of 2 M NaOH to obtain a carbohydrate concentration of 50 g L^{-1} and a NaOH

concentration of 0.1 M. Samples were stored in ice, before part of this solution was transferred to a 1.5 mL safe-lock tube and centrifuged in a CS-15R centrifuge (Beckman Coulter, Inc., Fullerton, CA) during 15 min at 4 °C and 14000*g* to remove undissolved material. After this centrifugation step, 500 μL of the supernatant was pipetted to a prewashed microcon YM 30 filter and centrifuged for 1 h and 45 min at 4 °C and 13000*g* to remove the enzyme. After centrifugation, 300 μL of the filtrate was taken and neutralized with 50 μL of 0.6 M HCl. This solution was analyzed with high-performance liquid chromatography (HPLC) to determine the carbohydrate composition. The HPLC column was an Aminex HPX-42A column (300 × 7.8 mm) from Bio-Rad (Veenendaal, The Netherlands) operated at 85 °C with a milli-Q water eluent at 0.3 mL/min. The amount of carbohydrates was determined with a refractive index detector. Calibration curves for glucose and maltose were approximately equal, and it was assumed that the calibration curves of maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were also the same. The weight fraction $x_{w,i}$ of a carbohydrate with a degree of polymerization (DP) equal to i was calculated with

$$x_{w,i} = \frac{\left(\frac{C_{DP_i}}{M_{w,DP_i}} i\right)}{\left(\frac{C_{tot}}{M_{w,g}}\right)} \times 100\% \quad (1)$$

where C_{DP_i} (in g/L) and M_{w,DP_i} (in g/mol) are respectively the concentration and molar mass of a malto-oligosaccharide, with a degree of polymerization equal to i, C_{tot} is the total carbohydrate concentration (in g/L), and $M_{w,g}$ is the molar mass of a D-glucopyranoside unit (162 g/mol). The total carbohydrate concentration was corrected for the increase in dry matter during the reaction caused by the formation of malto-oligosaccharides smaller than malto-octaose (for each malto-oligosaccharide that is formed, one molecule of water is used). Note that for the derivation of eq 1, the contribution of the molar mass of water ($M_{w,w}$) to the molar mass of carbohydrate polymers in native starch with the degree of polymerization n was neglected ($nM_{w,g} \gg M_{w,w}$). The carbohydrate composition was used to determine the yield and dextrose equivalent (DE) (for procedures, see ref *21*).

Model data were fitted to the experimentally determined weight fractions of oligosaccharides. The model equations were taken from ref *22* and fitted to our own data to make it easier to interpret the experimental data.

Measurement of the Enzyme Activity. For measurement of the residual enzyme activity, the grinded, frozen samples were dissolved in 0.1 M maleic acid buffer (final concentration of approximately 25 mg of enzyme solution L^{-1}) and stored in ice water, if it was not used directly for the enzyme activity assay. The Ceralpha end-point assay procedure, which was used to measure the α-amylase activity, is described elsewhere (*21*). In this case, the α-amylase activity is defined as the amount of *p*-nitrophenol that is released (in μmol mg^{-1} min^{-1} of enzyme stock solution) at 40 °C and pH 6.5 after hydrolysis of blocked *p*-nitrophenyl maltoheptaoside. Samples often contain various carbohydrates that affect the measurement of the α-amylase activity (*23, 24*). The experimentally determined α-amylase activity should be corrected for the presence of carbohydrates in the sample to obtain the true α-amylase activity. The method used to obtain the actual α-amylase activity is described in another paper (*21*).

RESULTS AND DISCUSSION

Effect of the Enzyme/Substrate Ratio on Enzymatic Hydrolysis. Three different enzyme/substrate ratios were used for the enzymatic hydrolysis of HT gelatinized wheat starch (experiments 3, 6, and 8 in **Table 1**). The highest enzyme/substrate ratio (10 w/w %) resulted in the highest dextrose equivalent (**Figure 1**). In addition, the initial rate of increase of the dextrose equivalent as function of time was largest for the highest enzyme/substrate ratio.

The initial slope of the dextrose equivalent versus time curve could be determined accurately at an enzyme/substrate ratio of 0.1 w/w %. In this case, the amount of substrate is in excess, and therefore, the initial hydrolysis rate should be investigated at this enzyme/substrate ratio. The high enzyme concentrations can be used to determine the maximum dextrose equivalent that can be reached with α-amylase from *B. licheniformis* at these reaction conditions.

The enzyme activity was also measured during the complete time course of these hydrolysis reactions. The scatter in these enzyme activity measurements was small and did not follow a pattern (see confidence intervals in **Table 1**), and for that reason, it was assumed that the amount of enzyme inactivation was negligible under these reaction conditions.

Effect of Starch Pretreatment on Enzymatic Hydrolysis. **Figure 2** shows the dextrose equivalent as function of time during the enzymatic hydrolysis of native, HP-gelatinized, and HT-gelatinized starches (experiments 1, 2, and 3 in **Table 1**, respectively). The initial slope of the dextrose equivalent versus time curve is comparable for native and HP-gelatinized starch in the first hour. After this period, the difference in the dextrose equivalent versus time curves of native and pressure-gelatinized starch starts to increase. The largest initial slope of the dextrose equivalent versus time curve was obtained with HT-gelatinized starch. The lowest dextrose equivalent is obtained after enzymatic hydrolysis of native starch, and the highest dextrose equivalent is obtained after hydrolysis of HT-gelatinized starch, while the dextrose equivalent of HP-gelatinized starch falls in between.

Table 1. Overview Experiments[a]

exp	C_e/C_s (w/w %)	T_G (°C)	P_G (MPa)	t_G (min)	T_H (°C)	P_H (MPa)	ν[b] (μmol mg^{-1} min^{-1})	EAP
1	0.1				50	0.1	1.41 ± 0.07[c]	
2	0.1	50	450	15	50	0.1	1.27 ± 0.05[c]	a
3	0.1	90	0.1	60	50	0.1	1.78 ± 0.17[c]	a
4	0.1	90	0.1	5	50	450[d]	1.40 ± 0.06[e]	a
5	1				50	0.1	1.11 ± 0.06[c]	
6	1	90	0.1	60	50	0.1	1.14 ± 0.03[c]	a
7	1	90	0.1	60	50	0.1	1.23 ± 0.05[f]	b
8	10	90	0.1	60	50	0.1	1.32 ± 0.06[c]	a

[a] Abbreviations: exp, experiment number; C_e/C_s, enzyme/substrate ratio; T_G, gelatinization temperature; P_G, gelatinization pressure; t_G, gelatinization time; T_H, hydrolysis temperature; P_H, hydrolysis pressure; ν, average enzyme activity; and EAP, enzyme addition point, with a and b standing for respectively the addition of the enzyme after and before gelatinization. [b] 95% confidence interval. [c] Average over 24 h. [d] Average over 6 h. [e] Average over 1.5 h. [f] During the first 15 min, the pressure was not equal to 450 MPa (approximately 12 min for sample preparation and 3 min for pressure buildup).

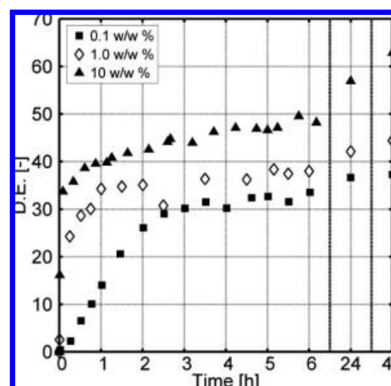

Figure 1. Dextrose equivalent as a function of the hydrolysis time for three different enzyme/substrate ratios after HT gelatinization (experiments 3, 6, and 8). Hydrolysis conditions: α-amylase from *B. licheniformis*, 50 °C, 5 w/w % wheat starch in water.

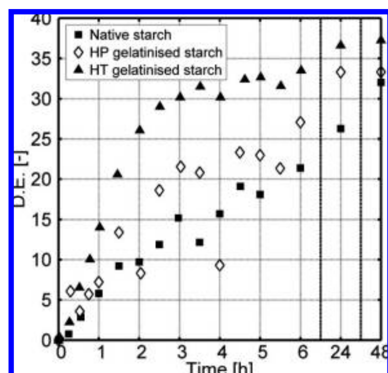

Figure 2. Dextrose equivalent as a function of hydrolysis time for native (experiment 1), HP-gelatinized (experiment 2), and HT-gelatinized (experiment 3) starches. Gelatinization conditions: HP = 450 MPa and 50 °C; HT = 0.1 MPa and 90 °C. Hydrolysis conditions: α-amylase from *B. licheniformis*, 50 °C, 5 w/w % wheat starch in water, enzyme/substrate ratio = 0.1 w/w %.

The enzyme was stable over the complete time course of the experiments with native, HP-gelatinized, and HP-gelatinized starch, as the scatter in the enzyme activity measurements was random and small (see confidence intervals in **Table 1**). As a consequence, differences between hydrolysis experiments with native, HP-gelatinized, and HT-gelatinized starches cannot be explained by considering the enzyme stability. Furthermore, the differences in the initial hydrolysis rate between native, pressure-gelatinized, and thermally gelatinized starch can also not be explained by differences in enzyme activity, because the ratio of the initial slopes (1.0:1.0:2.3) differs from the ratio of the enzyme activities (1.1:1.0:1.4).

Figure 3 shows the weight fractions of various oligosaccharides as a function of time during the experiments of which the dextrose equivalents are shown in **Figure 2**. Hydrolysis of native, HP-gelatinized, and HT-gelatinized starches results in similar weight fraction profiles for maltotetraose, maltopentaose, maltohexaose, and maltoheptaose. However, the weight fractions of these oligosaccharides during enzymatic hydrolysis are higher in the following order of used substrates: native < HP-gelatinized < HT-gelatinized starch. The weight fractions of maltose and maltotriose are approximately equal during the hydrolysis of HP-gelatinized starch. During the hydrolysis of HT-gelatinized starch, the maltotriose weight fraction is higher than the maltose weight fraction. Hydrolysis of native starch resulted in a maltose weight fraction that is higher than the maltotriose weight fraction. The weight fraction of glucose is lower than the weight fraction of maltose and maltotriose and of comparable magnitude in all cases. The yield of glucose, maltose, and maltotriose after 48 h of hydrolysis decreases in the following order: native starch (51%), HP-gelatinized starch (46%), and HT-gelatinized starch (44%). The yields of maltotetraose, maltopentaose, maltohexaose, and maltheptaose show the reverse trend (10, 26, and 44% for native, HP-gelatinized, and HT-gelatinized starch, respectively).

In case an enzyme/substrate ratio of 1.0 w/w % was used for enzymatic hydrolysis of native starch (experiment 5 in **Table 1**) instead of 0.1 w/w %, the dextrose equivalent and weight fractions were approximately the same as those in **Figure 2** and parts **C** and **D** of **Figure 3**, respectively. The yield of glucose, maltose, and maltotriose after 48 h of hydrolysis was 56%, while the yield of maltotetraose, maltopentaose, maltohexaose, and maltheptaose was 12%.

Studies in literature have shown that native starch can be hydrolyzed enzymatically (*25–28*). Guerrieri et al. (*26*) found

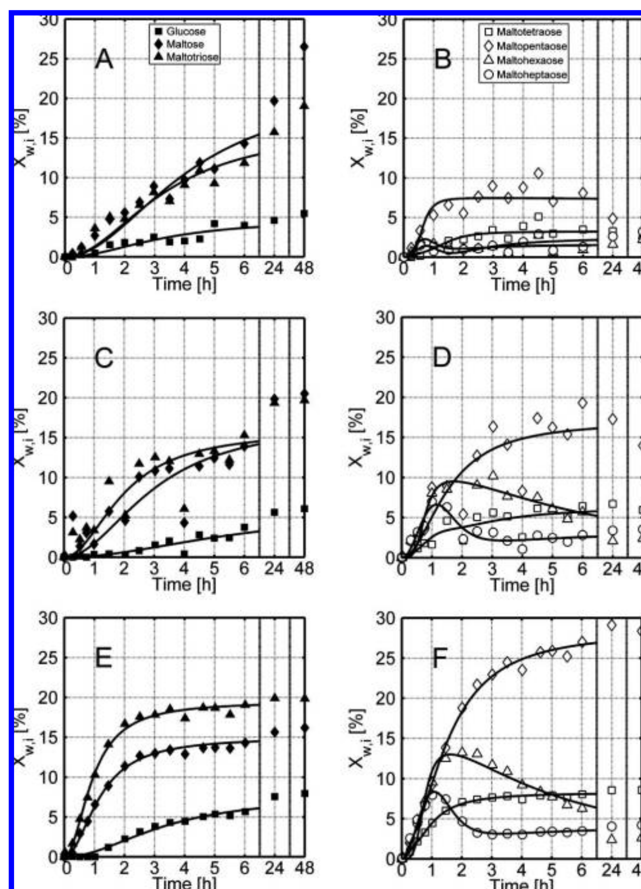

Figure 3. Weight fraction of several oligosaccharides as a function of time for native (**A** and **B**) (experiment 1), HP-gelatinized (**C** and **D**) (experiment 2), and HT-gelatinized (**E** and **F**) (experiment 3) starches. The lines were added to make it easier to interpret the experimental data. They are based on model equations from ref *22*. Gelatinization conditions: HP = 450 MPa and 50 °C; HT = 0.1 MPa and 90 °C. Hydrolysis conditions: α-amylase from *B. licheniformis*, 50 °C, 5 w/w % wheat starch in water, enzyme/substrate ratio = 0.1 w/w %.

that the use of native starch instead of temperature-gelatinized starch resulted in a much lower hydrolysis rate and final glucose yield during hydrolysis with amyloglucosidase at 60 °C. However, we observed that the differences between the initial hydrolysis rate (the initial slope of the dextrose equivalent versus time curve) and the final dextrose equivalent during the hydrolysis of native starch and HT-gelatinized starch with α-amylase from *B. licheniformis* were much smaller than the differences between native starch and HT-gelatinized starch that were observed by Guerrieri et al. (*26*). Perhaps α-amylase from *B. licheniformis* is able to hydrolyze native starches better than amyloglucosidase hydrolyses native starches because of a difference in the structure of these enzymes. Native starch is partly crystalline, and although these crystalline regions can be broken down (*29*), the hydrolysis rate is slow. According to Colonna et al. (*25*), the accessibility and crystallinity are the main limiting factors for the hydrolysis rate of native starch. Tester et al. (*28*) also state that the accessibility of the enzyme to the interior of the granules regulates the hydrolysis. When native starch granules are hydrolyzed, carbohydrates are slowly released or solubilized (*30*). As a result, the availability of carbohydrates is limiting during the hydrolysis of native starch and all carbohydrates that become available will be broken down rapidly by the enzyme (*30*). For that reason, the weight fractions of intermediates, such as oligosaccharides with a DP of 4–7,

are low and the weight fractions of end products, oligosaccharides with a DP of 1–3, increase only gradually during hydrolysis. Because the available substrate is the limiting factor during hydrolysis of native starch, an increase in the enzyme/substrate ratio should not affect the results. A comparison of the results of experiments 1 and 5 indeed showed that the weight fractions of small oligosaccharides (and therefore the yield and dextrose equivalent) did not change over the complete time course of the experiment.

During thermal gelatinization at 90 °C, granules gelatinize completely and become amorphous inside (*3, 6*). The carbohydrate chains are therefore much more accessible for the enzyme in comparison to native starch. Consequently, the number of accessible carbohydrate chains per enzyme molecule is so large that the enzyme concentration limits the hydrolysis rate. A higher enzyme/substrate ratio therefore leads to an increased hydrolysis rate (see **Figure 1**) and a faster increase in the oligosaccharide weight fractions. The enzyme has a preference for large carbohydrates (*23, 24*), and they are hydrolyzed first. After the large carbohydrates have been broken down into oligosaccharides, the oligosaccharides themselves are hydrolyzed. As a result of the large quantity of large carbohydrates, the weight fractions of oligosaccharides with a DP of 4–7 will remain relatively high during the course of the experiment in comparison to native starch. After some time, the large carbohydrates have been hydrolyzed and the oligosaccharides with a DP of 4–7 are the largest carbohydrates available for hydrolysis. The hydrolysis profiles shown in parts **E** and **F** of **Figure 3** agree with the results of Nakakuki et al. (*31*) and Saito (*32*) and the description given by Guzman-Maldonado and Paredes-Lopez (*33*).

The enzymatic hydrolysis of pressure-gelatinized starch falls in between the hydrolysis of native and heat-gelatinized starch. Consequently, it seems that the accessibility of the substrate of HP-gelatinized starch also falls in between that of HT-gelatinized and native starch. Douzals et al. (*34*), Stolt et al. (*35*), Katopo et al. (*36*), and Knorr et al. (*5*) mentioned that pressure-gelatinized granules do not disintegrate. In addition, double helices in amylopectin do not unwind during high-pressure treatment according to Knorr et al. (*5*). These two factors combined may lead to a lower accessibility of carbohydrates for the enzyme and, consequently, a lower amount of oligosaccharides with a DP of 4–7 when HP-gelatinized starch is hydrolyzed instead of HT-gelatinized starch. Our results agree with the results of Hayashi and Hayashida (*14*), who found that hydrolysis of pressure-treated starch instead of native starch resulted in a higher hydrolysis yield when α-amylase from *Bacillus* sp. was used. In addition, Stute et al. (*16*) found that the degree of hydrolysis with amyloglucosidase was slightly lower when pressure-gelatinized starch (550 MPa) was hydrolyzed instead of thermally gelatinized starch, which agrees with our results obtained with our enzyme. However, Selmi et al. (*15*) found that the use of pressure-gelatinized starch instead of thermally gelatinized starch resulted in a higher glucose production yield during hydrolysis with amyloglucosidase. They suggest that this difference can be explained by structural differences between high-pressure- and high-temperature-gelatinized starches and the presence of amylose−lipid complexes formed during thermal gelatinization. Although the rate at which these amylose−lipid complexes are broken down might be low (*37*), they can be hydrolyzed enzymatically (*37*) (*38*) (*39*). As a result, we expect that the presence of amylose−lipid complexes cannot lead to a lower glucose yield, it can only result in a slower increase of the glucose production in time.

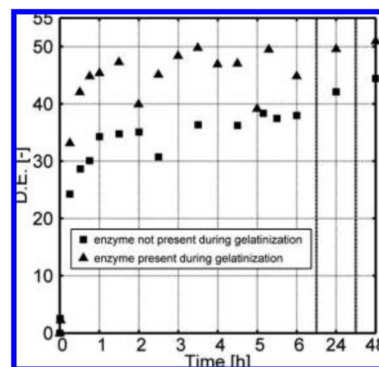

Figure 4. Dextrose equivalent as a function of the starch hydrolysis time when the enzyme was present during gelatinization (experiment 7) and when the enzyme was added after gelatinization (experiment 6). $t = 0$ is the point at which the enzyme was added. Gelatinization conditions: 0.1 MPa and 90 °C. Hydrolysis conditions: α-amylase from *B. licheniformis*, 50 °C, 5 w/w % wheat starch in water, enzyme/substrate ratio = 1.0 w/w %.

Hydrolysis of native, HP-gelatinized, and HT-gelatinized starch resulted in different oligosaccharide versus time profiles. These differences were probably caused by differences in the crystallinity and ordering, leading to differences in the specific surface area (area/volume ratio) of the substrate. After high-temperature gelatinization, starch is present as a slurry of carbohydrate polymers surrounded by water with a large specific area, making these carbohydrates easily accessible for the enzyme. However, native starches consist of granules that consist of alternating amorphous and semicrystalline growth rings, in which the carbohydrates are only present on the outside of the granules and via the pores into the granules, leading to a smaller specific area and a low accessibility for the enzyme. After high-pressure gelatinization, part of the structure has been lost but the granular shape is still intact, leading to a situation that is comparable to the one encountered with native starch. However, after high-pressure gelatinization, the granules have also taken up water and become amorphous inside. Because of the uptake of water, swelling occurred and the diameter of the granules increased, leading to a higher surface area. This might explain why the hydrolysis of pressure-gelatinized starch is faster than the hydrolysis of native starch. Apparently, limited accessibility because of a low specific surface area results in a more gradual release of carbohydrates that are immediately converted to glucose, maltose, and maltotriose by excess quantities of enzyme.

Effect of the Enzyme Addition Point on Enzymatic Hydrolysis. Figure 4 shows the dextrose equivalent as a function of time in case the enzyme is absent or present during gelatinization. With the enzyme present during gelatinization at 90 °C (experiment 7 in **Table 1**), the dextrose equivalent was higher (see **Figure 4**) than the dextrose equivalent obtained after hydrolysis of starch gelatinized at the same temperature in the absence of enzyme (experiment 6 in **Table 1**). **Figure 5** shows the weight fractions of various oligosaccharides in time for these experiments. In both cases, the weight fraction profiles of maltose, maltotetraose, maltohexaose, and maltoheptaose were similar. However, the weight fractions of glucose, maltotriose, and maltopentaose were much higher when the enzyme was already present during gelatinization. Enzymatic hydrolysis of native cassava starch at 80 °C (*22*), which also results in gelatinization and hydrolysis at the same time, resulted in similar oligosaccharide profiles, as we found in parts **C** and **D** of **Figure 5**.

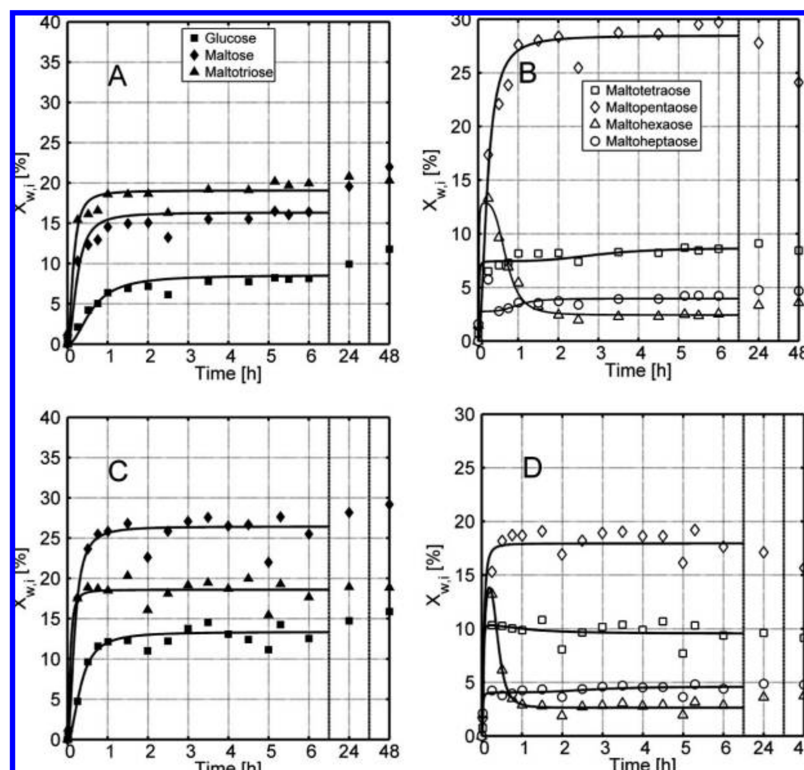

Figure 5. Weight fractions of several oligosaccharides as a function of time when the enzyme was added after gelatinization (**A** and **B**) (experiment 7) and when the enzyme was present during gelatinization (**C** and **D**) (experiment 6). The lines were added to make it easier to interpret the experimental data. They are based on model equations from ref *22*. Gelatinization conditions: 0.1 MPa and 90 °C. Hydrolysis conditions: α-amylase from *B. licheniformis*, 50 °C, 5 w/w % wheat starch in water, enzyme/substrate ratio = 1.0 w/w %.

Enzyme deactivation did not take place during the first 6 h of these experiments, because the scatter in the enzyme activity measurements was random and small (see confidence intervals in **Table 1**). After 24 and 48 h, the residual enzyme activity was equal to, respectively, 90 and 60% in case the enzyme was present during gelatinization, while no enzyme deactivation could be observed if the enzyme was added after gelatinization.

During gelatinization at 90 °C, the initial enzyme activity is approximately 40% higher than at 50 °C (*11*). Our measurements indicated that the enzyme was also stable at these conditions (probably because of the presence of substrate) during the time used for gelatinization. Because of the high activity and high stability at 90 °C, a higher hydrolysis rate is expected, resulting in a higher dextrose equivalent as observed. However, we also observed a difference in the oligosaccharide weight fractions of experiments 6 and 7. If this difference is only caused by a higher reaction rate and not by a change in the hydrolysis mechanism, a higher enzyme concentration (experiment 8) should lead to similar results, as observed for experiment 7. The weight fractions of all oligosaccharides during experiment 8 (results not shown) are comparable to those observed in parts **C** and **D** of **Figure 5**. However, the weight fraction of maltose is lower, and the weight fraction of maltopentaose is higher. As a result, the higher hydrolysis rate cannot explain our results.

Our results might be related to differences in substrate accessibility during gelatinization in the presence of the enzyme. In the beginning of the process, the starch granules are not yet gelatinized, because the reaction mixture is first heated from 20 to 90 °C (30 min). Consequently, enzymatic hydrolysis proceeds according to the hydrolysis of native starch (as shown in parts **A** and **B** of **Figure 3**) as long as the temperature in the reaction mixture is below the gelatinization onset temperature (approximately 50 °C). Two aspects that confirm this hypothesis are the high glucose and maltose weight fractions and the low maltopentaose weight fraction. During heating, the accessibility of carbohydrates for the enzyme increases. Consequently, the hydrolysis starts to proceed according to the hydrolysis of HP-gelatinized starch (as shown in parts **C** and **D** of **Figure 3**). This is confirmed by the high weight fractions of oligosaccharides with a DP of 4–7. When the temperature has become 90 °C, which is sufficient to gelatinize starch completely, the hydrolysis proceeds according to the hydrolysis of HT-gelatinized starch (parts **E** and **F** of **Figure 3**). Note that the combination of a high temperature during gelatinization and a high enzyme/substrate ratio decreases the time to reach a more or less stable carbohydrate composition from 2 to 1 h of hydrolysis. Additional proof for the hypothesis based on substrate accessibility was found when experiment 7 was repeated with a gelatinization time of 5 min instead of 60 min (results not shown). Although the gelatinization time was much shorter, the weight fractions of the measured oligosaccharides were the same during the complete time course of the experiment.

When the enzyme was present during gelatinization at 450 MPa and 50 °C, starch sedimented during gelatinization. As a result, it is possible that a more dense solid starch phase is formed where, only at the interface, sufficient enzyme is present for starch hydrolysis, and the results of this experiment are therefore not reliable.

Effect of Pressure on Enzymatic Hydrolysis. In **Figure 6**, the results are shown for the hydrolysis of HT-gelatinized starch that was hydrolyzed at ambient pressure (experiment 3 in **Table 1**) and 450 MPa (experiment 4 in **Table 1**). **Figure 6A** shows that the initial increase of the dextrose equivalent in time was approximately the same for both conditions. Parts **B** and **C** of **Figure 6** show that the initial increase of the weight fractions

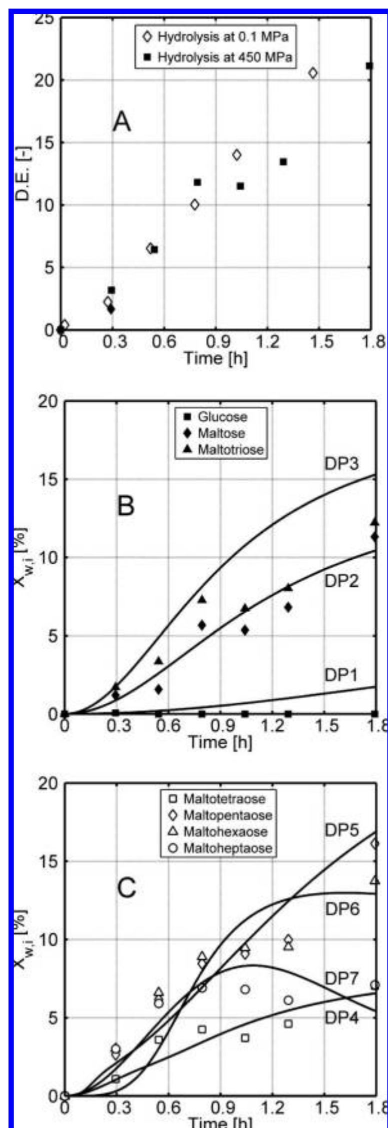

Figure 6. Dextrose equivalent **(A)** and mole fractions of several oligosaccharides (**B** and **C**) as a function of time when HT-gelatinized starch was hydrolyzed at 450 MPa and 50 °C (experiment 4). The experimental dextrose equivalent **(A)** and the lines from parts **E** and **F** of **Figure 3** for mole fractions of several oligosaccharides found during the hydrolysis of HT-gelatinized starch at atmospheric pressure and 50 °C were added (experiment 3). Gelatinization conditions: 0.1 MPa and 90 °C. Additional hydrolysis conditions: α-amylase from *B. licheniformis*, 5 w/w % wheat starch in water, enzyme/substrate ratio = 0.1 w/w %.

of all measured oligosaccharides was approximately the same when a hydrolysis pressure of 450 MPa was used instead of 0.1 MPa. The weight fractions of all other measured oligosaccharides are comparable during the experiment, except for glucose and maltotriose. The weight fractions of these carbohydrates are lower when the enzymatic hydrolysis is carried out at 450 MPa instead of 0.1 MPa. Additional experiments are required to draw more definite conclusions about the selectivity on a larger time scale. On the basis of our results, it seems that the use of 450 MPa instead of atmospheric pressure during hydrolysis does not lead to significantly different hydrolysis products. For that reason, additional experiments were not pursued.

During hydrolysis at 450 MPa, we found that the enzyme was stable over the complete time course of the experiment, which agrees with the results obtained by Weemaes at al. (*10*). The enzyme activity measurements showed some (random) scatter, but the variability was small (see confidence intervals in **Table 1**).

Consequences for Process Design. The pretreatment of starch and the enzyme addition point strongly influence the hydrolysis rate and the weight fractions of oligosaccharides that are formed. In case the weight fraction of oligosaccharides with a DP of 4–7 should be low in comparison to the weight fraction of nonhydrolyzable carbohydrates, native starch should be used as a substrate. In addition, the ratio between glucose, maltose, and maltotriose can be altered by choosing different process conditions. Hydrolysis of native starch can be used for obtaining the highest maltose weight fraction (it seems at the cost of longer reaction times and lower conversion), while the hydrolysis of HT-gelatinized starch can be used to yield the lowest maltose weight fraction. Having α-amylase present during gelatinization resulted in higher glucose and maltose weight fractions and a lower weight fraction of maltopentaose as compared to the hydrolysis where the enzyme was added after gelatinization. Approximately the same weight fractions of oligosaccharides with a DP of 4, 5, and 7 were obtained.

The hydrolysis rate can also be affected by the process. In case high-weight fractions of all oligosaccharides with a DP of 1−7 are required in a short period of time, HT-gelatinized starch should be used (either in the presence or absence of enzyme). It is therefore used during liquefaction (*1, 40*) to supply partially hydrolyzed carbohydrates for subsequent saccharification with other enzymes. In case the amount of glucose, maltose, and maltotriose should increase gradually in time, native starch should be used as a substrate. Hydrolysis of native starch might be combined with fermentation to provide a steady-state supply of fermentable carbohydrates (glucose, maltose, and maltotriose) to a yeast to avoid inhibition by these small carbohydrates (*41*).

During one of the gelatinization experiments, α-amylase from *B. licheniformis* was present during gelatinization. This enzyme is stable at high temperatures and high pressures. Many starch-converting enzymes are less stable at high temperatures or high pressures. However, stability measurements are usually carried out in the absence of substrate, while the presence of substrate results in a higher stability (*42*) because of the stabilization of the enzyme by the substrate. In addition, we have used 60 min to gelatinize starch in the presence of the enzyme. However, the gelatinization conditions can be chosen in such a way that complete gelatinization is reached in a shorter period of time, thus further minimizing the amount of enzyme deactivation. If, for example, glucoamylase is used to hydrolyze starch, the starch gelatinization diagram of Douzals et al. (*3*) together with the activity measurements of Buckow et al. (*43*) can be used to select the conditions (pressure, temperature, and treatment time) that result in complete gelatinization while limiting or even preventing enzyme deactivation. Finally, the stability of the enzyme can be increased using a higher substrate concentration (*6*), enabling the user to retain complete enzyme activity during gelatinization.

ABBREVIATIONS USED

DE, dextrose equivalent; DP, degree of polymerization; HP, high-pressure treatment (450 MPa, 50 °C); HPLC, high-performance liquid chromatography; HT, high-temperature treatment (0.1 MPa, 90 °C).

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