Bacillus stearothermophilus Neopullulanase Selective Hydrolysis of Amylose to Maltose in the Presence of Amylopectin

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The specificity of Bacillus stearothermophilus TRS40 neopullulanase toward amylose and amylopectin was analyzed. Although this neopullulanase completely hydrolyzed amylose to produce maltose as the main product, it scarcely hydrolyzed amylopectin. The molecular mass of amylopectin was decreased by only one order of magnitude, from approximately $10^6$ to $10^7$ Da. Furthermore, this neopullulanase selectively hydrolyzed amylose when starch was used as a substrate. This phenomenon, efficient hydrolysis of amylose but not amylopectin, was also observed with cyclomaltodextrinase from alkalophilic Bacillus sp. strain A2-5a and maltogenic amylase from Bacillus licheniformis ATCC 27811. These three enzymes hydrolyzed cyclomaltodextrins and amylose much faster than pullulan. Other amylolytic enzymes, such as bacterial saccharifying α-amylase, bacterial liquefying α-amylase, β-amylase, and neopullulanase from Bacillus megaterium, did not exhibit this distinct substrate specificity at all, i.e., the preference of amylose to amylopectin.

We previously found a new type of pullulan-hydrolyzing enzyme, neopullulanase (EC 3.2.1.135) from Bacillus stearothermophilus TRS40 (15), and showed that it catalyzes the hydrolysis of α-1,4- and α-1,6-glucosidic linkages (6), as well as transglycosylation to form α-1,4- and α-1,6-glucosidic linkages (27). The replacement of several amino acid residues that constitute the active center of the neopullulanase showed that one active center of the enzyme participated in all four of the reactions described above (17). Based on this series of experimental results using the neopullulanase and the structural similarities of the enzymes that catalyze these four reactions, we proposed and defined a general idea for one enzyme family, an α-amylase family (27). Based on the concept of the α-amylase family (13), we controlled the substrate preference and transglycosylation activity of the neopullulanase (14), and Preiss and his coworkers (16) analyzed the regions that determined the specificity of maize branching enzyme (EC 2.4.1.18) isoforms.

The neopullulanase hydrolyzes pullulan to produce panose (Glc pα1-6Glc pα1-4Glc) as the main product, Maltose and glucose are also produced as the final products. The final molar ratio of panose, maltose, and glucose is about 3:1:1 (6). Neopullulanases have been reported for other bacteria, such as Bacteroides thetaiotaomicron (25), alkalophilic Bacillus (5), and Bacillus polymyxa CECT155 (33). Most of the α-amylases that have been investigated so far could not hydrolyze pullulan. However, neopullulanase-type α-amylase has also been reported for Thermoactinomyces vulgaris (31). Maltogenic amylase (EC 3.2.1.133) from Bacillus licheniformis ATCC 27811 also hydrolyzed pullulan to produce panose (9).

Starch constitutes most of the dry matter in certain crops, and it therefore is not only the primary source of calories in the human diet but can also be regarded as a renewable resource that can be used in many industrial applications. It is a mixture of two macromolecules: amylose, which is essentially composed only of α-1,4-linked glucose polymers, and amylopectin, which is composed of α-1,4-linked glucose polymers branched by α-1,6 linkages. The possibility that amylolytic enzymes may have different specificities toward amylose and amylopectin has been discussed previously (8, 15).

We describe here a unique macromolecule recognition by B. stearothermophilus neopullulanase in comparison with other amylolytic enzymes. The similar substrate preferences of a cyclomaltodextrinase (EC 3.2.1.54) and a maltogenic amylase are also reported.

**MATERIALS AND METHODS**

**Chemicals.** Synthetic amyloses with average molecular masses of 70, 110, 320, and 1,000 kDa (amyloses AS-70, -110, -320, and -1000, respectively) were purchased from Nakano Vinegar Co., Ltd. (Aichi, Japan). Amylose EX-1 (average degree of polymerization, 17), amylose EX-III (average degree of polymerization, 117), and pullulan (average molecular mass, 96 kDa) were purchased from Hayashibara Biochemical Laboratories Inc. (Okayama, Japan). Highly branched cyclic dextrin was prepared in our laboratory (28). Amylose and amylopectin from potato starch were purchased from Sigma Chemical Co. (St. Louis, Mo.). Potato starch and waxy maize starch were obtained from Nihon Shokuhin Kako Co. Ltd. (Tokyo, Japan). Cyclomaltodextrins, soluble starch, and other chemical reagents were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

**Enzymes.** The neopullulanase was purified from the culture broth of Bacillus subtilis ANA-1 (21) (arg-15 hsdR hsdM Δapra3 amyE npr) carrying pPP10 (Te′ nptI′ [structural gene of the neopullulanase from B. stearothermophilus TRS40]) as described previously (15). Bacterial liquefying α-amylase from Bacillus licheniformis was purchased from Sigma. Bacterial saccharifying α-amylase from B. subtilis was from Nagase Chemtex (Osaka, Japan). Maltogenic α-amylase from B. stearothermophilus (Novamyl) (1) was obtained from Novozymes (Tokyo, Japan).

**Enzyme assays.** The activities of the amylolytic enzymes were assayed using potato amylose as a substrate. The reaction mixture (200 μl) containing 0.5% potato amylose in buffer (described below) and the enzyme was incubated at 40°C (cyclomaltodextrinase, B. licheniformis maltogenic amylase, and B. megaterium neopullulanase) or 50°C (B. stearothermophilus neopullulanase, bacterial...
saccharifying α-amylase, bacterial liquefying α-amylase, Novamyl, and β-amylase) for 10 min. The buffer contained 100 mM sodium acetate buffer (pH 5.5) for bacterial liquefying α-amylase, bacterial saccharifying α-amylase, and β-amylase; 100 mM sodium acetate buffer (pH 5.0) for Novamyl; 100 mM sodium 2-(N-morpholino)ethanesulfonic acid (MES)-Na buffer (pH 6.0) for B. stearothermophilus neopullulanase and B. megaterium neopullulanase; and 100 mM sodium phosphate buffer (pH 6.5) for cyclomaltodextrinase and B. licheniformis maltogenic amylase. The reducing sugar after the enzyme reaction was assayed based on the 3,5-dinitrosalicylic acid method as described previously (22). One unit of enzyme activity was defined as the amount of enzyme needed to produce 1 μmol of reducing sugar as glucose per min from potato amyllose under the assay conditions described above.

Expression of the cyclomaltodextrinase in Escherichia coli. The cyclomaltodextrinase gene was amplified by PCR from chromosomal DNA ofalkalophilic Bacillus sp. strain A2-5a (23). The forward primer 5'-TGGCCTACGTGAAAAGAAACGATTTA-3' introduces an Ncol site (underlined) at the authentic ATG codon and is located between positions 9060 and 9079 of the cyclomaltodextrinase gene (GenBank accession no. AB015670). The reverse primer 5'-TCTGCTATCCTTAAATCACCCTTTATAACACC-3' introduces a KpnI site (underlined) and is located between positions 9217 and 9236 of the gene. These primers generate mutations at the second codon from TTA to GTA and is located between positions 2010 and 2027 of the gene. The amplification product was digested with Ncol and KpnI and then introduced into the Ncol-KpnI site in pKKS81-1 (Clontech, Palo Alto, Calif.) to obtain the expression plasmid pNP963. E. coli TG-1 (supE hsdS thy Δ(lac-proAB)F- [m13d6 proAB lacZ Δ(lacI-ΔM15]) carrying pNP963 was grown in Terrific broth (Gibco BRL) containing 50 μg of ampicillin per ml at 37°C until the late log phase. The culture was induced with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) and the culture was incubated for 20 h at 15°C. Cells were harvested and washed with 10 mM sodium phosphate buffer (pH 7.5) (buffer A) and disrupted by sonication in buffer A at 4°C. Cell debris was removed from the crude extract by centrifugation, and the enzyme was precipitated by adding solid (NH₄)₂SO₄ to 80% saturation. The precipitate was redissolved and dialyzed against buffer A. The culture supernatant was collected and the enzyme was precipitated by adding solid (NH₄)₂SO₄ to 80% saturation. The precipitate was redissolved and dialyzed against buffer A. The active fraction was not absorbed by the column. The culture solution was again passed through the column, concentrated using polyethylene glycol (molecular mass, 20,000) and stored at 4°C.

Expression of the maltogenic amylase gene in E. coli. The maltogenic amylase gene was amplified by PCR from chromosomal DNA of B. licheniformis ATCC27811. The forward primer 5'-TGGCCTATGATCGAATTCGACCGA TAC-3' introduces an Ndel site (underlined) upstream of the start codon and is located between positions 292 and 313 of the maltogenic amylase gene (GenBank accession no. X67133). The reverse primer 5'-CTTTAATGTTGGCATCACAGAATTTACGGCCG-3' introduces an EcoRI site (underlined), changes the stop codon from TTAG to TAA, and is located between positions 2020 and 2027 of the gene. The amplified fragment was digested with Ndel and EcoRI and then introduced into the Ndel-EcoRI site in pGEX-Nde2 to obtain the expression plasmid pNP422. The expression vector pGEX-Nde2 is a derivative of pGEX-5X-3 (Amersham) and was constructed by introducing four nucleotides into the unique XbaI site of pGEX-Nde (30).

E. coli TG-1 carrying pNP422 was grown in Terrific broth containing 50 μg of ampicillin per ml at 37°C until the late log phase. IPTG was then added to a final concentration of 0.1 mM, and the culture was incubated for 20 h at 15°C. Cells were harvested and washed with buffer A and disrupted by sonication in buffer A at 4°C. Cell debris was removed from the crude extract by centrifugation, and the enzyme was precipitated by adding solid (NH₄)₂SO₄ to 70% saturation. The precipitate was redissolved and dialyzed against buffer A. The dialysate was loaded onto a G-Sepharose Fast Flow column equilibrated with buffer A. The active fraction was not absorbed by the column. The culture solution was again passed through the column, concentrated using polyethylene glycol (molecular mass, 20,000) and stored at 4°C.

Measurement of amylase or amylopectin contents during neopullulanase action on starch. Amylose was fractionated from the reaction mixture by a modification (4) of the method of Lansky et al. (18) and purified by recrystallization from aqueous 10% 1-butanol. Amylopectin was fractionated by precipitation by adding a sevenfold volume of ethanol after removing the amylose fraction. The low-molecular-mass products, which contain maltose as a main component, remained in the supernatant after the precipitation of amylopectin. The amylose and amylopectin fractions were dissolved in 90% dimethyl sulfoxide (DMSO). The total carbohydrate contents of these three fractions were measured by the phenol-sulfuric acid method (2). The degradation ratio was calculated from the contents of the reducing sugar as maltose per total carbohydrate. The content of reducing sugar was measured by the Somogyi-Nelson method (26).

Expression of the maltogenic amylase gene in B. megaterium. The maltogenic amylase gene was cloned and expressed in B. megaterium according to a published method (23). The maltogenic amylase gene nucleotide sequence (GenBank accession no. X07261) and is located between positions 1158 and 1176. The reverse primer 5'-TTCATAGTAAAAGGGAAATGTGACAG-3' introduces an Spel site (underlined) upstream of the second codon of the B. megaterium neopullulanase gene sequence (GenBank accession no. X07261) and is located between positions 1158 and 1176. The amplified gene was digested with Spel and BclI, separated on an agarose gel, and cloned into the Spel-BclII site in the E. coli-Bacillus shuttle vector pWH152 (Mobitec GmbH) to obtain the expression plasmid pNP78. This plasmid encodes a fusion protein of the four N-terminal amino acids of the XylaA protein of pWH152 and B. megaterium neopullulanase.

B. subtilis ANA1 (arg-15 hisD16 lacproA3 amyE3 ypr) carrying pNP78 was grown at 37°C in L broth (1% Tryptone [Difco], 0.5% yeast extract [Difco], 0.5% NaCl [pH 7.3]) containing 20 μg of tetracycline per ml. At the mid log phase, xylose was added to a final concentration of 0.5% to induce expression of the B. megaterium neopullulanase gene. After 22 h, the culture supernatant was collected and the enzyme was precipitated by adding solid (NH₄)₂SO₄ to 80% saturation. The precipitate was redissolved and dialyzed against buffer A. The dialysate was loaded onto a Q-Sepharose Fast Flow column equilibrated with buffer A. The active fraction was not absorbed by the column. The enzyme solution was again passed through the column, concentrated using polyethylene glycol (molecular mass, 20,000), and stored at 4°C.

Measurement of amylase or amylopectin action on amylose. Amylose was fractionated from the reaction mixture by a modification (4) of the method of Lansky et al. (18) and purified by recrystallization from aqueous 10% 1-butanol. Amylopectin was fractionated by precipitation by adding a sevenfold volume of ethanol after removing the amylose fraction. The low-molecular-mass products, which contain maltose as a main component, remained in the supernatant after the precipitation of amylopectin. The amylose and amylopectin fractions were dissolved in 90% dimethyl sulfoxide (DMSO). The total carbohydrate contents of these three fractions were measured by the phenol-sulfuric acid method (2). The degradation ratio was calculated from the contents of the reducing sugar as maltose per total carbohydrate. The content of reducing sugar was measured by the Somogyi-Nelson method (26).

Measurement of the molecular mass of each fractionated product from amylose or amylopectin. The molecular mass was determined by high-performance liquid chromatography with a multilayer-laser light-scattering photometer (MALLIS) (DAWN DSP; Wyatt Technology Co., Ltd., Santa Barbara, Calif.) and a differential RI detector (RI-6A; Shimadzu, Kyoto, Japan).
RESULTS

Degradation of amylose or amylopectin with B. stearothermophilus neopullulanase and other amylolytic enzymes. The 1% amylose and 1% amylopectin (both from potato starch) solutions were prepared from the 10% substrates solutions in 90% DMSO by dilution with deionized water. The diluted solution was used immediately for the enzyme reaction. The substrate solution (300 μl) was individually mixed with enzyme solutions (300 μl each) that contained equal amounts of activity (12 U) toward potato amylose. Bacterial liquefying -amylase, bacterial saccharifying -amylase, and -amylase were solubilized in 200 mM sodium acetate (pH 5.5); Novamyl was solubilized in 200 mM sodium acetate buffer (pH 5.0); and B. stearothermophilus neopullulanase, Novamyl, β-amylase, bacterial saccharifying α-amylase, and bacterial liquefying α-amylase, respectively. The reaction times (minutes) are indicated below the lanes.

FIG. 1. Thin-layer chromatograms of reaction products of amylolytic enzymes from amylose (A) or amylopectin (B). Lane M, standard maltooligosaccharides; G1, G2, G3, G4, G5, and G6, glucose, maltose, maltotriose, maltotetraose, maltopentaose, and maltohexaose, respectively. NPL, NOV, β-AMY, BSA, and BLA, B. stearothermophilus neopullulanase, Novamyl, β-amylase, bacterial saccharifying α-amylase, and bacterial liquefying α-amylase, respectively. The reaction times (minutes) are indicated below the lanes.

FIG. 2. Time course of the reaction of B. stearothermophilus neopullulanase on a mixture of amylopectin (peak A) and amylose (peak B). The elution profile was analyzed by gel permeation chromatography.

lose and amylopectin and produced the same products from each. Although B. stearothermophilus neopullulanase efficiently hydrolyzed amylose to produce maltose and a small amount of glucose, it scarcely hydrolyzed amylopectin (Fig. 1). These results indicate that the specificity of B. stearothermophilus neopullulanase toward amylose and amylopectin was clearly different from those of other amylolytic enzymes.

Confirmation of the selective hydrolysis of amylose by B. stearothermophilus neopullulanase. Synthetic amylose (amylose AS-70) or amylopectin (from potato starch) solutions were prepared from the 10% substrate solutions in 90% DMSO by dilution with deionized water. The diluted solution was used immediately for enzyme treatment. A reaction mixture (1.5 ml) consisting of 0.5% synthetic amylose, 0.5% potato amylopectin, and B. stearothermophilus neopullulanase (0.6 U) in 100 mM Mes-Na buffer (pH 6.0) was incubated at 50°C. After incubation for 0, 10, 30, 60, and 120 min, 270-μl samples were collected, and the reaction was terminated by heat treatment at 100°C for 5 min. After removal of the precipitate by centrifugation, the supernatant (250 μl) was subjected to gel permeation chromatography. Since synthetic amylose with an average molecular mass of 70 kDa and potato amylopectin were used as the substrates, these two components were clearly fractionated by column chromatography, as shown in Fig. 2.
Although the peak of amylose gradually disappeared upon treatment with *B. stearothermophilus* neopullulanase, the peak of amylopectin was not affected even after 120 min. This result indicates that *B. stearothermophilus* neopullulanase selectively hydrolyzes amylose even in a mixture of amylose and amylopectin.

**Action of B. stearothermophilus neopullulanase on starch.**

Potato starch, consisting of about 20% amylose and 80% amylopectin, was solubilized by heating and used as a substrate for *B. stearothermophilus* neopullulanase. A reaction mixture (10 ml) consisting of 1.0% potato starch and *B. stearothermophilus* neopullulanase (40 U) in 100 mM MES-Na buffer (pH 6.0) was incubated at 50°C. After incubation for 0, 5, 10, 20, 30, 60, 120, 180, 270, and 1,440 min, 1-ml samples were collected, and the reaction was terminated by heat treatment at 100°C for 5 min. After removal of the precipitate by centrifugation, the supernatant was used to analyze the contents of amylose, amylopectin, and low-molecular-mass fractions. These three fractions were obtained by using 1-butanol as described in Materials and Methods. Figure 3 shows the contents of these fractions during the action of *B. stearothermophilus* neopullulanase on potato starch. The amylose fraction gradually decreased with enzyme treatment, while the amylopectin fraction was not significantly affected during the reaction period. The low-molecular-mass fraction increased in response to the decrease in the amylose fraction. The degradation rate was calculated from the amount of reducing sugar as maltose per total carbohydrate, and it was assumed that the low-molecular-mass fraction was mainly maltose and was produced only from the amylose fraction (Fig. 3). The results indicated that *B. stearothermophilus* neopullulanase selectively hydrolyzed amylose even in native starch.

The time courses of the changes in the molecular masses of the amylose and amylopectin fractions during the *B. stearothermophilus* neopullulanase reaction were analyzed by high-performance liquid chromatography with MALLS and RI (Fig. 4). The molecular mass of amylose rapidly decreased below $10^5$ Da within 120 min, and the amylose fraction was not observed after 120 min (Fig. 3). Therefore, it is most likely that the amylose was completely hydrolyzed to maltooligosaccharides, mainly maltose. The molecular mass of the native amylopectin from potato was greater than $10^6$ Da. Although the molecular mass of potato amylopectin slightly decreased to approximately $10^5$ Da, it was not reduced further by *B. stearothermophilus* neopullulanase (Fig. 4). Even when an excess amount of the enzyme (400 U, 10 times the amount used in the case described above) was used for the same reaction period (1,440 min), the same phenomenon was observed. Similar results were observed in experiments using starches from other sources, such as tapioca, maize, rice, and wheat.

The change in the chain length distribution of amylopectin after treatment with *B. stearothermophilus* neopullulanase was investigated by HPAEC (Fig. 5). There was little difference in chain length distribution between the neopullulanase-treated amylopectin and unreacted amylopectin.

**Comparison of the substrate specificities of B. stearothermophilus neopullulanase and other amylolytic enzymes.**

The initial velocities of the reactions of *B. stearothermophilus* neopullulanase and other amylolytic enzymes with various substrates were measured quantitatively (Table 1). Reaction mixtures (200 μl) containing 0.5% substrates and enzyme were incubated at 40°C (cyclomaltodextrinase, *B. licheniformis* maltogenic amylase, and *B. megaterium* neopullulanase) or 50°C (*B. stearothermophilus* neopullulanase, bacterial saccharifying α-amylose, bacterial liquefying α-amylose, Novamyl, and β-amylose) for 10 min. Potato amylose, synthetic amylose, maltooligosaccharides, amylopectin, waxy maize starch, highly branched cyclic dextrin, potato starch, cyclomaltodextrins, and

![FIG. 3. Time courses of the contents of amylose, amylopectin, and low-molecular-mass fractions during the reaction of *B. stearothermophilus* neopullulanase on potato starch. The contents of amylopectin (stippled bars), amylose (gray bars), and low-molecular-mass (white bars) fractions are shown against the amount of total carbohydrate. The degradation ratio in each reaction period is shown as a percentage of reducing sugar as maltose per total carbohydrate.](http://aem.asm.org/)

![FIG. 4. Time courses of the molecular masses of the amylopectin (□) and amylose (●) fractions during the reaction of *B. stearothermophilus* neopullulanase on potato starch. The molecular mass was measured by high-performance liquid chromatography with the MALLS system.](http://aem.asm.org/)
pullulan were used as the substrate for the enzyme reaction. The same buffer as in the enzyme assay was used for each enzyme. The same amount of each enzyme (0.76 U) was used, based on potato amylose-hydrolyzing activity.

The substrate specificity of B. steaothermophilus neopullulanase was similar to those of cyclomaltodextrinase and B. licheniformis maltogenic amylase but was quite different from those of the other amylolytic enzymes (Table 1). B. steaothermophilus neopullulanase, cyclomaltodextrinase, and B. licheniformis maltogenic amylase efficiently hydrolyzed amylose, maltotriose, and maltodextrins but scarcely hydrolyzed amylopectin. On the other hand, the other amylolytic enzymes did not exhibit significantly different specificities toward amylose and amylopectin. B. megaterium neopullulanase was secreted in the culture broth of B. subtilis ANA-1 carrying pNPR78 (with the B. megaterium neopullulanase gene) as described in Materials and Methods. Since B. megaterium neopullulanase hydrolyzed pullulan (Table 1) to produce panose (data not shown), it could be classified as a neopullulanase (33). However, B. megaterium neopullulanase did not exhibit a significantly different specificity toward amylose and amylopectin.

FIG. 5. Chain length distribution of amylopectin after treatment with B. steaothermophilus neopullulanase. A reaction mixture (1.5 ml) consisting of 0.5% potato amylopectin and B. steaothermophilus neopullulanase (0.6 U) in 100 mM Mes-Na buffer (pH 6.0) was incubated at 50°C. After incubation for 0, 60, and 270 min, 270-μl samples were collected, and the reaction was terminated by heat treatment at 100°C for 5 min. The products were completely debranched by isoamylase as described previously (29). The solutions of the debranched products (25 μl; 100 μg each) were analyzed by HPAEC. The numbers with dots are degrees of polymerization.

DISCUSSION

We found a unique macromolecule recognition by the neopullulanase from B. steaothermophilus TRS40. Although B. steaothermophilus neopullulanase hydrolyzed amylose to produce maltose and a small amount of glucose (Fig. 1), it scarcely hydrolyzed amylopectin (Fig. 1 and 2). Furthermore, it selectively hydrolyzed amylose when starch was used as the substrate (Fig. 3). While the molecular mass of amylose decreased sharply below 10^6 Da, that of amylopectin decreased by only one order of magnitude, from approximately 10^6 to 10^5 Da (Fig. 4). There was little difference in the chain length distribution between the neopullulanase-treated amylopectin and unreacted amylopectin (Fig. 5). Other amylolytic enzymes such as α-amylase and β-amylase did not exhibit this distinct substrate specificity (Fig. 1; Table 1).

Amylose and cyclomaltodextrins were good substrates for B. steaothermophilus neopullulanase (Table 1). It is speculated that the helical structure of amylose and the pseudohelical structure of cyclomaltodextrins might fit into the active center of the enzyme. Amylose is a relatively small, linear molecule of 5 × 10^5 to 1 × 10^6 Da, and amylopectin is a much larger (1 × 10^7 to 1 × 10^8 Da), branched molecule with a large spherical shape. Based on the observation of a polymodal distribution of chain lengths in amylopectin, a cluster model has been suggested (3). Based on the chain lengths (3), the packing model of chains (7), and the size of clusters derived from electron microscopic analysis (32), the molecular mass of each cluster is calculated as being on the order of 10^7 Da (28). However, the amylopectin molecule is organized at a further large level of structure that has been called a blocklet (11). The structure of amylopectin at the level higher than the cluster has not been fully understood. Since the degradation of amylopectin completely halted at a molecular mass of approximately 10^7 Da, a structure susceptible to the action by B. steaothermophilus neopullulanase may exist at an interval of every several tens of clusters in amylopectin. Indeed, B. steaothermophilus neopullulanase did not hydrolyze waxy maize starch, which is composed only of amylopectin with a molecular mass of approximately 10^7 Da (data not shown), or highly branched cyclic dextrin, which is composed of a cluster structure with a molecular mass of approximately 5 × 10^5 Da (28) (Table 1).

This unique macromolecule recognition by B. steaothermophilus neopullulanase was also observed in the reactions of cyclomaltodextrinase and B. licheniformis maltogenic amylase (Table 1). These three enzymes exhibit 40 to 60% amino acid sequence identity (24). There is presently great interest in the structure-function relationships of these enzymes (19). Although B. megaterium neopullulanase hydrolyzed pullulan to produce panose, it did not exhibit distinct substrate specificity toward amylose and amylopectin (Table 1). Since neopullulanase from B. steaothermophilus TRS40 and that from B. megaterium NCIMB11568 have less than 30% sequence identity, B. megaterium neopullulanase and the neopullulanase from B. polymyxa CECT155 (33) can be classified into different categories. In this context, B. steaothermophilus neopullulanase, cyclomaltodextrinase, and B. licheniformis maltogenic amylase are intracellular enzymes, while B. megaterium neopullulanase and B. polymyxa neopullulanase are extracellular enzymes (10). The problem of identification and the physiological roles of the
enzymes in the α-amylase family have been discussed previ-
ously (19, 24).

A partially degraded starch, dextrin, of various sizes is widely
used in the food and chemical industries. A new type of dextr-
in, highly branched cyclic dextrin, with a molecular mass of
approximately $5 \times 10^7$ Da was recently produced in our lab-
oratory (28). We also produced a new dextrin with a molecular
mass of approximately $10^7$ Da which was 20 times larger than
highly branched cyclic dextrin, using Bacillus stearothermophilus
neopullulanase. Some characteristics of this new dextrin have
been investigated. The viscosity of the dextrin paste was much
lower than that of gelatinized intact starch paste. The new
dextrin solution also has a low propensity for retrogradation. Bacillus
stearothermophilus neopullulanase may also be used to produce
low-amylase or amylase-free starch. Investigation of the indus-
trial application of the unique properties of this enzyme is now
in progress.

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