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Purification and characterisation of a malto-oligosaccharide-forming amylase active at high pH from *Bacillus clausii* BT-21

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Abstract

Bacillus clausii BT-21 produced an extracellular malto-oligosaccharide-forming amylase active at high pH when grown on starch substrates. The enzyme was purified to homogeneity by affinity and anion-exchange chromatography. The molecular weight of the enzyme estimated by sodium dodecyl sulfate polyacrylamide electrophoresis was 101 kDa. The enzyme showed an optimum of activity at pH 9.5 and 55 °C. Maltohexaose was detected as the main initially formed starch hydrolysis product. Maltotetraose and maltose were the main products obtained after hydrolysis of starch by the enzyme for an extended period of time and were not further degraded. The enzyme readily hydrolysed soluble starch, amylopectin and amylose, while cyclodextrins, pullulan or dextran were not degraded. The mode of action during hydrolysis of starch indicated an *exo*-acting type of amylolytic enzyme mainly producing maltohexaose and maltotetraose. Amino acid sequencing of the enzyme revealed high homology with the maltohexaose-forming amylase from *Bacillus* sp. H-167.

Keywords: *Bacillus clausii* BT-21; Malto-oligosaccharide-forming amylase; Malto-oligosaccharides

1. Introduction

Malto-oligosaccharides are composed of 2–10 units of α-D-glucopyranose linked by an α-(1→4) bond. These compounds have potential applications in the food industry due to their properties such as low sweetness, high waterholding capacity, and prevention of sucrose crystallisation [1]. The preparation of malto-oligosaccharides with a degree of polymerisation (DP) above 3 in larger amounts is however tedious and expensive. The discovery of microbial enzymes, which predominantly formed malto-oligosaccharides of a specific length allows the production of larger amounts of these oligosaccharides [2].

Amylases are starch-degrading enzymes classified as hydrolases, which cleave α-D-(1→4) O-glucosidic linkages in starch. All α-amylases (E.C. 3.2.1.1, α-D-(1→4)-glucan glucanohydrolase) are *endo*-acting enzymes cleaving α-D-(1→4) O-glucosidic linkages within the starch molecule in a random fashion [3]. In contrast, the *exo*-acting amylolytic enzymes, such as β-amylases (E.C. 3.2.1.2, α-D-(1→4)-

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glucan maltohydrolase), and some malto-oligosaccharide-forming amylases cleave the starch molecule from the non-reducing end of the substrate [4]. β-Amylases, α-glucosidases (E.C. 3.2.1.20, α-D-glucoside glucohydrolase), glucoamylase (E.C. 3.2.1.3, α-D-(1→4)-glucan glucohydrolase), and malto-oligosaccharide-forming amylases can produce malto-oligosaccharides of a specific length from starch.

Several amylases producing malto-oligosaccharides of a specific DP have been identified previously including maltohexaose-producing amylases from *Klebsiella pneumonia* [5,6], *B. subtilis* [7], *B. circulans* G-6 [8], *B. circulans* F-2 [9,10], and *B. caldovelox* [11,12]. Maltopentaose-producing amylases have been detected in *B. licheniformis* 584 [13] and *Pseudomonas* spp. [14,15]. Furthermore, maltotetraose-producing amylases have been reported from *Pseudomonas stutzeri* NRRL B-3389 [16,17], *Bacillus* sp. MG-4 [18] and *Pseudomonas* sp. IMD353 [19] and maltotriose-producing amylases from *Streptomyces griseus* NA-468 [20] and *B. subtilis* [21].

Very few malto-oligosaccharide-forming amylases active at high pH have been identified previously including those from *Bacillus* sp. H-167 producing maltohexaose [22,23], from a bacterial isolate (163-26, DSM 5853) producing maltopentaose [24], from *Bacillus* sp. IMD370 producing maltotetraose and smaller malto-oligosaccharides [25], and from *Bacillus* sp. GM 8901 initially producing maltohexaose from starch which was converted to maltotetraose during extended hydrolysis [26].

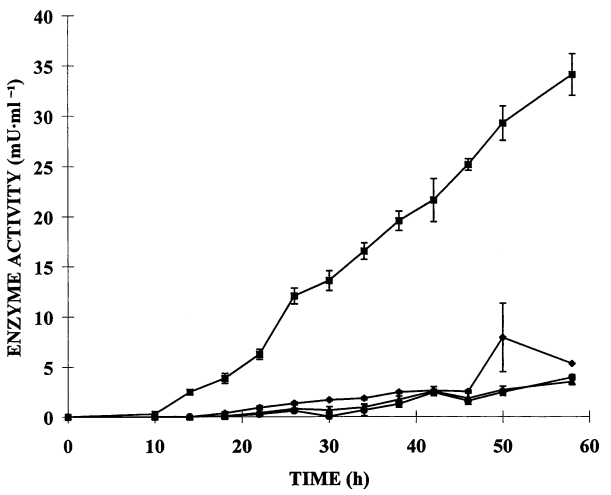

Fig. 1. Extracellular amylolytic activity (mU mL^{-1}) in liquid cultures of *B. clausii* BT-21 grown on 2% starch substrates at 45 °C. ◆ Soluble potato starch, ● amylopectin, ▲ corn starch, ■ whole brown rice. Bars indicate the standard deviation.

In this paper we report the purification and characterisation of a malto-oligosaccharide-forming *exo*-acting amylase active at high pH isolated from an alkali-tolerant strain of *Bacillus clausii* BT-21.

2. Results and discussion

Identification of B. clausii *BT-21.*—According to its fatty acid composition, the strain showed similarity to the genus *Bacillus*. A partial sequencing of the 16S-rDNA showed a similarity of 99.4% to *B. clausii*. The physiological properties of the alkali-tolerant strain confirmed this identification.

Production of amylase activity by B. clausii *BT-21.*—Growth in liquid cultures containing soluble potato starch, corn starch, amylopectin from corn or whole brown rice resulted in different levels of extracellular amylase activity in the medium. The soluble (up to 50 $g \cdot L^{-1}$ at 90 °C) potato starch is composed of amylose (30%) and amylopectin (70%) with phosphate mono ester groups linked per 300 glucose units of the amylopectin. Corn starch contains more lipids and no phosphorus, and amylopectin is highly branched containing α-D-(1→6) O-glycosidic linkages. These three types of starch are readily accessible for hydrolysis after heat gelatinisation, while whole brown rice contains a less accessible starch encapsulated in the rice grains. The amylase activities detected in the extracellular fluid of liquid cultures with the different starch substrates are shown in Fig. 1. The highest amylolytic activity was obtained with whole brown rice as a substrate. Similar results were obtained with wheat bran, which was however difficult to remove from the extracellular fluid prior to the purification of the enzyme. Carbon sources such as galactose, glycogen, and inulin have previously been reported as suitable substrates for the production of amylases by *B. licheniformis* [27] and soluble starch has been found as the best substrate for the production of an amylase by *B. stearothermophilus* [28]. However, none of

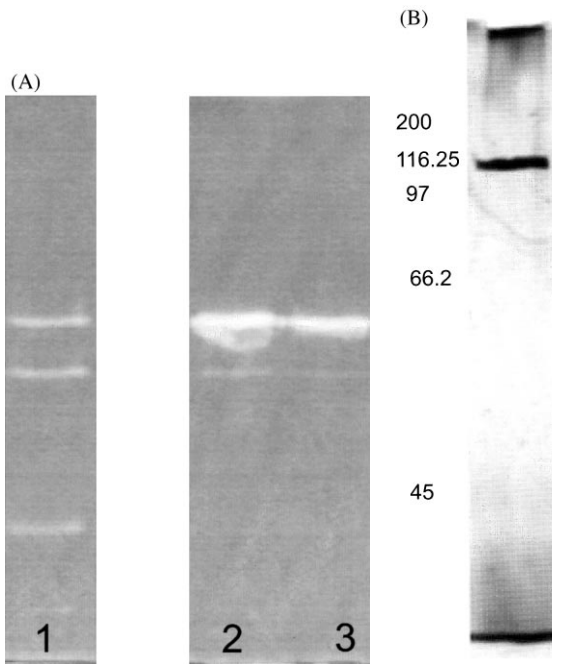

Fig. 2. (A) Detection of amylolytic activities after different purification steps by native PAGE (10%). Lane 1: extracellular fluid; lane 2: after β-CD affinity chromatography; lane 3: after anion-exchange chromatography. (B) 10% SDS-PAGE of the purified enzyme preparation after anion-exchange chromatography and of molecular weight markers.

these studies have included less accessible starch substrates.

Purification of the malto-oligosaccharide-forming amylase.—The enzyme was purified by batch affinity chromatography with β-CD sepharose 6B followed by anion-exchange chromatography (Table 1). Analysis of amylase activity in different enzyme fractions following native tris-glycine PAGE (polyacrylamide gel electrophoresis) indicated the presence of 3 amylolytic activities in the extracellular fluid (Fig. 2(A, lane 1)). One of the enzymes could be separated from the other amylolytic activities by a β-CD affinity and an anion-exchange chromatography step (Fig. 2(A), lanes 2 and 3, respectively). SDS-PAGE of the purified enzyme preparation indicated that the enzyme had been purified to homogeneity and had an apparent molecular weight of approximately 101 kDa (Fig. 2(B)). Cyclodextrin sepharose 6B affinity chromatography has been previously applied for the purification of an α-amylase as a final purification step after removal of other amylases by anion-exchange chromatography [29]. The enzyme recovery of 8.7% and the purification factor of 18.5 obtained for the malto-oligosaccharide-forming amylase were similar to the results reported by these authors.

Characterisation of the malto-oligosaccharide-forming amylase.—The purified enzyme showed an optimum of activity at pH 9.5 (Fig. 3(A)), while the optimum temperature for its activity was found at 55 °C (Fig. 3(B)). The enzyme showed between 55 and 70 °C higher activity in the presence of 5 mM $CaCl_2$. The stability of the enzyme at pH 9.5 in the presence of 5 mM $CaCl_2$ after a 30 min incubation period at different temperatures is shown in Fig. 3(C). At temperatures above 55 °C, the enzyme lost 75% of its maximum activity. Five other malto-oligosaccharide-forming amylases with an alkaline pH optimum preferentially forming maltohexaose [23], maltopentaose [24] and maltotetraose [26] have been previously reported. They also showed a similar temperature optimum of about 55 °C. The molecular weights of these enzymes have been estimated at 59, 73 and 80 kDa [23], 180 kDa [24] and 97 kDa [26]. Most of the previously reported malto-oligosaccharide-forming amylases and α-amylases showed a lower molecular weight in the range of 50–65 kDa [3,16,17,19,21].

The distribution of products detected by high performance anion-exchange chromatography (HPAEC) after hydrolysis of soluble potato starch for various time intervals by the purified enzyme is shown in Fig. 4. After 2 h of incubation, the amount of maltohexaose, and after 4 h the amount of maltopentaose decreased while the amounts of maltotetraose, maltotriose, maltose and glucose increased. These products accumulated after prolonged hydrolysis times indicating that they were not further hydrolysed. After 24 h of starch hydrolysis, the amounts of malto-oligosaccharides formed were 3% maltohexaose, 4% maltopentaose, 41% maltotetraose, 13% maltotriose, 16% maltose and 4% glucose. Similar results have been obtained in a time course study of the hydrolysis of soluble starch by the malto-oligosaccharide-forming amylases from *Bacillus* sp. H-167 [23] and *Bacillus* sp. GM8901 [26]. These amylases also produced maltohexaose preferentially in the early stage of hydrolysis. Kim et al. [26] found that the initial hydrolysis product of a maltotetraose-

Table 1
Purification of the malto-oligosaccharide-forming amylase from *B. clausii* BT-21

	Volume (mL)	Activity (mU mL^{-1})	Total activity (mU)	Total protein (mg)	Specific activity (mU mg^{-1} protein)	Recovery (%)	Purification factor
Extracellular fluid	4,000	155	620,000	424	731	100	1
β-CD affinity chromatography	704.5	88	62,137	8.5	3,647	10.0	5
Dialysis and concentration	172.6	254	43,840	4.8	4,581	7.1	6.3
Anion-exchange chromatography	215	251	54,051	2.0	13,493	8.7	18.5

forming amylase after 1 h hydrolysis of starch was mainly maltohexaose (54%) followed by a gradual increase in the amounts of maltotetraose and maltose while the amount of maltohexaose decreased. After 20 h, the composition of malto-oligosaccharides had changed to 0.6% maltohexaose, 1.3% maltopentaose, 53.2% maltotetraose, 8.3% maltotriose, 27.6% maltose and 9% glucose.

The enzyme readily hydrolysed soluble potato starch, amylose and amylopectin. The hydrolysis products formed from 1% soluble potato starch or 1% amylopectin after 4 h of incubation with enzyme (67 U·mL^{-1}) were maltotetraose (2%), maltopentaose (28%) and maltohexaose (70%). The hydrolysis of amylose (0.1% w/v) resulted in higher amounts of maltotetraose (19%), maltotriose (10%) and glucose (4%), while less maltopentaose (16%) and maltohexaose (51%) were formed.

The enzyme did not hydrolyse pullulan, a glucan composed of maltotriose units linked by α-(1→6) O-glucosidic linkages. This result indicates that similar to the malto-oligosaccharide-forming amylase from *P. stutzeri* [16], it could not bypass the α-(1→6) O-glucosidic linkages next to three glucose units and thereby cleave this substrate. Dextran, a glucan containing α-(1→6) O-glucosidic linkages, as well as α-, β-, and γ-cyclodextrins, cyclic malto-oligosaccharides composed of 6, 7, and 8 glucose units, were not hydrolysed by the enzyme. The main initial starch hydrolysis product detected after 30 min reaction by the amylase from *B. clausii* BT-21 was maltohexaose, as shown in Fig. 5. Starch hydrolysis products larger than DP 6 could not be detected pointing to the presence of an *exo*-type action mode of the enzyme.

Plotting the decrease of the blue colour of a starch–iodine complex caused by the cleavage of starch against the amount of reducing sugars released by the enzyme [30] clearly indicated the presence of an *exo*-acting enzyme similar to the maltotetraohydrolase from *P. stutzeri* (Fig. 6). In contrast, an *endo*-acting α-amylase from *Aspergillus oryzae* produced a much steeper curve.

The hydrolysis of malto-oligosaccharides by the purified enzyme was also examined. Maltose, maltotriose, and maltotetraose were not

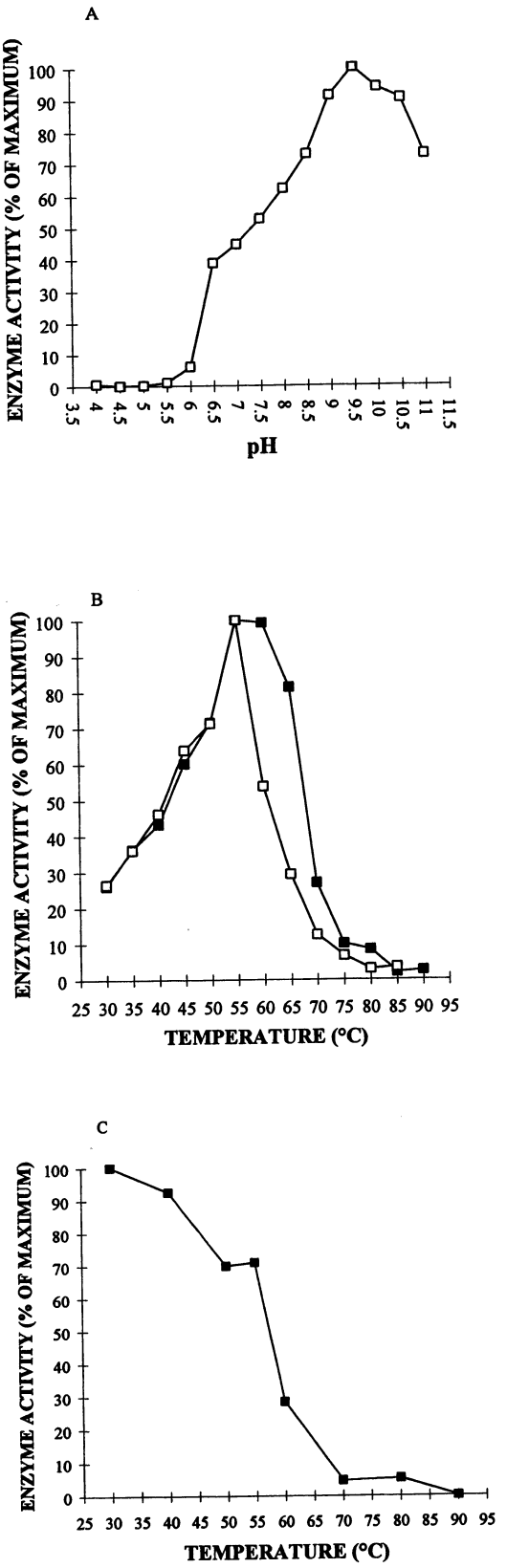

Fig. 3. Effect of pH and temperature on the activity of the malto-oligosaccharide-forming amylase. (A) Effect of pH at 55 °C. (B) Effect of temperature at pH 9.5 ■ with 5 mM $CaCl_2$ □ without $CaCl_2$. (C) Thermostability (residual activity after incubation for 30 min) at pH 9.5 with 5 mM $CaCl_2$.

hydrolysed by the *B. clausii* BT-21 amylase and transglycosylation products were not detected. This confirms the results obtained with soluble potato starch, where these compounds were detected as end products of the hydrolysis. A time course of the hydrolysis of a mixture of malto-oligosaccharides with DP 4–10 by the enzyme showed initially a rapid increase in the amount of maltohexaose and a slower increase in the amount of maltopentaose and maltotetraose (Fig. 7). The amounts of maltose and maltotriose slowly increased and the amounts of malto-oligosaccharides with DP 7–10 decreased. The compounds with a higher DP were hydrolysed faster than the ones with a lower DP. During prolonged times of incubation, the amount of maltohexaose declined while the amount of maltotetraose further increased.

For amino acid sequencing, the amylase was purified by a modified method including hydrophobic interaction and affinity chromatography. The purified preparation yielded a protein band with the same apparent molecular weight of 101 kDa according to SDS-PAGE as obtained by affinity and anion-exchange chromatography. However, native tris-glycine PAGE of the preparation followed by amylase activity staining or silver staining revealed the presence of two bands. The starch hydrolysis products formed by the enzyme preparations obtained by the two purification methods were identical. By direct sequencing of the amylase preparation we obtained the N-terminal sequence SQNSDQKLFSWENATVYFAIT. After tryptic digestion the following four peptide sequences were obtained: DMNQYGFGNPGVTEDWTPXEGQ, DAANEALWSWR, LVDGGTYLMLLPGGVK, and VVLDVVMNHPGYNTIK with X indicating that no residue has been assigned. The latter three of the tryptic peptides were completely sequenced and their sequences could be verified by comparing their molecular weights detected by MS with those predicted from their sequences.

The obtained sequences show more than 90% identity with the maltohexaose-producing amylase of *Bacillus* sp. H-167 [22,23,31]. This amylase has a similar molecular weight (102

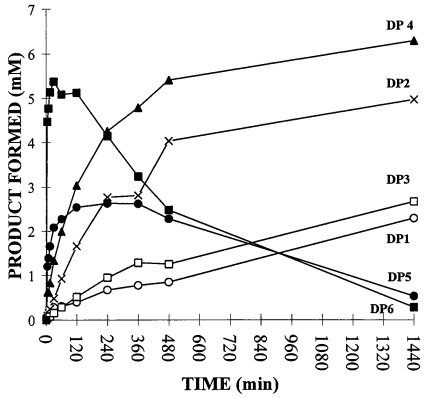

Fig. 4. Amounts of malto-oligosaccharides from DP 1 to 6 formed during hydrolysis of 1% soluble starch by the purified amylase (505 mU mL^{-1}) at 55 °C and pH 9.5. ○ glucose (DP 1), × maltose (DP 2), □ maltotriose (DP 3), ▲ maltotetraose (DP 4), ● maltopentaose (DP 5), ■ maltohexaose (DP 6).

598 Da) as the purified *B. clausii* BT-21 amylase. The maltohexaose-forming amylase of *Bacillus* sp. H-167 is proteolytically processed at the C-terminus into three isoforms [22,23,31], which could also explain the presence of two isoforms with apparently the same N-terminal sequence in *B. clausii* BT-21. While the *Bacillus* sp. H-167 amylase was reported to have an *endo*-type of action [22], our results obtained by measuring the hydrolysis of a starch–iodine complex clearly indicated that the *B. clausii* BT-21 amylase is an *exo*-acting type of enzyme.

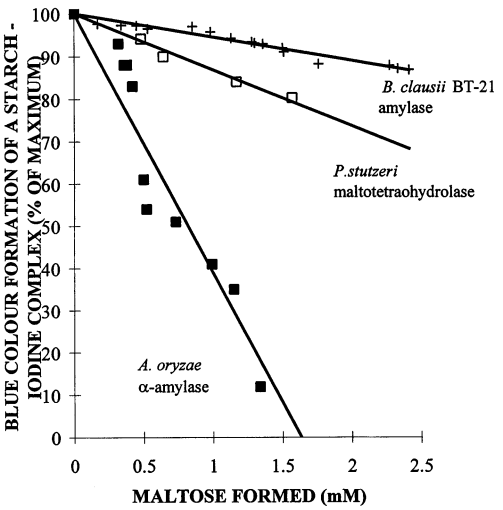

Fig. 6. Determination of *endo*- and *exo*-activity of the malto-oligosaccharide-forming amylase from *B.clausii* BT-21 compared with the maltotetraohydrolase from *P. stutzeri* and an α-amylase from *A. oryzae*.

The results also suggested that the hydrolysis of starch by the malto-oligosaccharide-forming amylase from *B. clausii* BT-21 proceeded in two steps consisting of an initial degradation of starch to mainly maltohexaose and to a lesser degree maltopentaose, which were further hydrolysed to mainly maltotetraose and maltose after extended hydrolysis times. A careful control of the enzymatic hydrolysis conditions is therefore necessary to obtain maximum yields of maltohexaose as a product of starch hydrolysis by this enzyme.

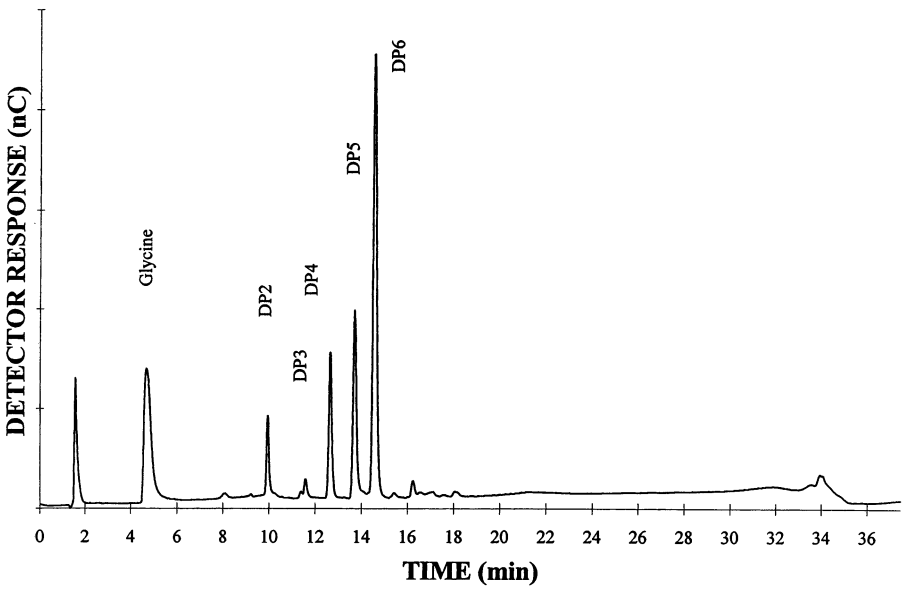

Fig. 5. HPAEC of products obtained by incubating the malto-oligosaccharide-forming amylase (505 mU mL^{-1}) for 30 min with 1% soluble potato starch at pH 9.5 and 55 °C. Glycine, internal standard.

3. Experimental

Materials.—Amylopectin and amylose from corn, corn starch, carboxymethylcellulose (CMC), bovine serum albumin (BSA), dextran, pullulan, maltose, maltotriose and a mixture of maltotetraose to maltodecaose were obtained from Sigma Chemical Co., St. Louis, USA. Soluble potato starch was obtained from E. Merck KGaA, Darmstadt, Germany. Yeast extract and tryptone were obtained from Difco Laboratories, Detroit, USA. Whole brown rice from Neue Allgemeine Reisgesellschaft mbH, Hamburg, Germany was used. Pharmaceutical grade α-, β-, and γ-cyclodextrins were obtained from Wacker Chemie Danmark Aps, Glostrup, Denmark. α-Amylase from *A. oryzae* was obtained from Novo Nordisk A/S, Bagsvaerd, Denmark. Maltotetraose was prepared as described previously [32]. All chemicals were, unless stated otherwise, of analytical grade.

Isolation of B. clausii *BT-21.*—The strain was isolated from compost material from Assens, Denmark and was identified by DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany).

Production of the enzyme.—*B. clausii* BT-21 was grown in an optimised liquid medium composed of 2.0% soluble potato starch, amylopectin from corn, corn starch, or whole brown rice, 0.5% yeast extract, 0.5% tryptone, 0.1% KH_2PO_4, 0.1%, Na_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% $CaCl_2 \cdot 2H_2O$, and 0.1% $(NH_4)_2SO_4$. After autoclaving, a sterile Na_2CO_3 solution was added to a final concentration of 1% (approximately pH 10). A 1.0 mL spore suspension in glycerol (stored at −80 °C) was used to inoculate 100 mL of medium and incubated at 45 °C for 18 h in a shaking incubator (New Brunswick Scientific, Edison, NJ, USA) at 200 rpm. Two mL of this culture were used to inoculate a shake flask with 200 mL medium and incubated at 45 °C in a shaking incubator. Aliquots were taken at regular intervals and the OD at 600 nm was measured to determine the growth of the strain in the media. Samples (4 mL) were centrifuged at 9600 rpm for 10 min at 4 °C and the pH and amylase activity was determined. All growth experiments were carried out in triplicate. The mean value ($X = (\Sigma_{i=1}^{n} X_i)/n$) and the standard deviation (std = $(\Sigma_{i=1}^{n}(X_i - X)/(n-1))$ was determined.

Purification of the malto-oligosaccharide-forming amylase.—After growth of *B. clausii* BT-21 on whole brown rice for 52 h, the cells and the whole rice grains were removed from the extracellular fluid (1 L) by centrifugation at 9600 rpm for 15 min at 4 °C. The malto-oligosaccharide-forming amylase was purified using an affinity gel prepared by covalently binding β-cyclodextrin to an epoxy-activated sepharose 6B matrix (Pharmacia Biotech, Uppsala, Sweden) [33]. The extracellular cell-

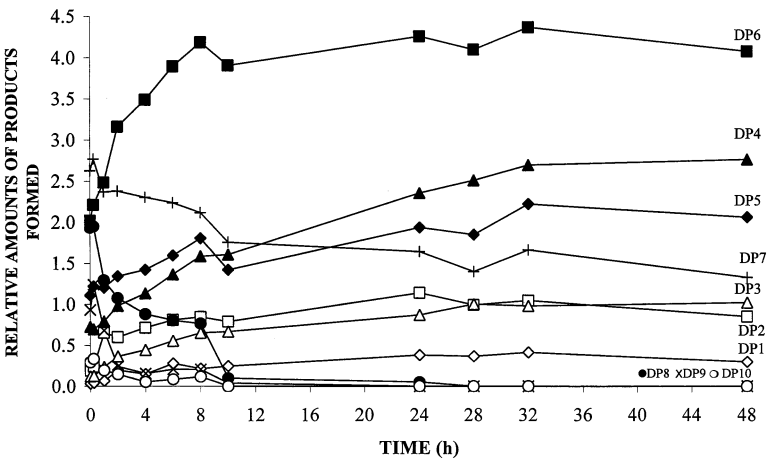

Fig. 7. Products detected by HPAEC after incubating the purified amylase (147 mU mL^{-1}) with a mixture of malto-oligosaccharides (DP 4–10, 5 mM) in borate buffer (pH 9.5) containing 5 mM $CaCl_2$ at 55 °C. ◇ DP 1, glucose; □ DP 2, maltose; △ DP 3, maltotriose; ▲ DP 4, maltotetraose; ◆ DP 5, maltopentaose; ■ DP 6, maltohexaose; + DP 7, maltoheptaose; ● DP 8, maltooctaose; × DP 9, maltononaose; ○ DP 10, maltodecaose.

free supernatant was incubated with 12 g of gel while shaking for 1 h at 4 °C. The supernatant was then removed by centrifugation at 9600 rpm for 10 min at 4 °C. Unbound protein was removed by washing the gel with 75 mL 50 mM phosphate buffer pH 8.0 followed by centrifugation. The washing step was repeated seven times. Bound protein was eluted with 45 mL of 50 mM phosphate buffer pH 8.0 containing 10 mM α-cyclodextrin followed by centrifugation. The elution step was repeated four times. α-Cyclodextrin was used for elution of the enzyme, since β- and γ-cyclodextrin interfered with the protein determination method [33]. The α-cyclodextrin was then removed by dialysis (6–8 kDa Spectra/Por dialysis membrane, The Spectrum Companies, Gardena, CA, USA) against 5 L 10 mM triethanolamin pH 7.5 while stirring at 4 °C. The buffer was changed after 2 h followed by an additional 12 h of dialysis. The dialysis bags were placed in CMC to concentrate the sample. Samples (10 mL) were applied to a HiTrap Q column (5 mL prepacked, Pharmacia) using a FPLC-system (Pharmacia). The proteins were eluted at the rate of 1.0 mL min^{-1} with 25 mL 10 mM triethanolamin pH 7.5 followed by a gradient of 20 mM NaCl min^{-1} in 10 mM triethanolamin pH 7.5. The enzyme was eluted at 0.5 M NaCl. The protein content was estimated by the method of Bradford [34] using the BIO-RAD Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). BSA was used as standard.

A modified purification method was used to isolate the enzyme for amino acid sequencing. The cell-free supernatant was fractionated using hydrophobic interaction chromatography by loading 100 mL supernatant adjusted to 0.5 M sodium sulfate onto a 70 mL column of Phenyl Sepharose low substitution (Pharmacia) at 5 mL min^{-1}. Unbound protein was washed off the column with 220 mL 50 mM triethanolamine 2 mM calcium chloride, 0.5 M sodium sulfate, pH 7.2 and bound protein was eluted with a gradient of 0.5–0 M sodium sulfate in 50 mM triethanolamine, 2 mM calcium chloride, pH 7.2 over 30 min at 5 mL min^{-1}. The amylase-containing fractions were pooled (80 mL) and loaded onto a 16 mL column of β-cyclodextrin coupled Sepharose 6B prepared as described above. The column was washed with 48 mL of 50 mM triethanolamine, 2 mM calcium chloride, pH 7.2 and eluted with 56 mL of the same buffer with 20 mM α-cyclodextrin. The amylase-containing fractions of the elute were pooled and used for sequencing.

Gel electrophoresis.—Samples (15 μL) were analysed using a 10% native tris-glycine PAGE gel [35]. The gel was after electrophoresis placed in 50 mM phosphate buffer at pH 6.5 and shaken for 30 min. A 1% soluble potato starch solution was incubated with the gel while shaking for 45 min. After washing in buffer solution, the gel was incubated with an iodine solution (4 mM I_2, 160 mM KI) and decoloured with buffer. Destained bands indicated amylase activity.

SDS-PAGE (10%) was performed according to [36] followed by silver staining [37]. A SDS-PAGE broad range molecular weight standard (Bio-Rad) was used.

Enzyme assay.—Two mL soluble potato starch solution (1.25%) in 0.1 M borate buffer pH 10.0 was incubated with 0.5 mL enzyme solution for 2 h at 45 °C. The reaction was stopped by boiling the mixture for 10 min.

The formation of reducing sugars was determined with the $CuSO_4$–bicinchonate assay [38] and calculated as mM maltose formed. One unit of activity corresponded to the amount of enzyme that produced 1 μmol maltose min^{-1} at pH 10.0 and 45 °C.

The decrease of the blue colour of a starch–iodine complex by enzymatic hydrolysis [39] was measured in duplicates spectrophotometrically at 600 nm. Properly diluted enzyme solutions (2.5 mL) were incubated with 10.0 mL soluble potato starch (1.25%) in buffer (borate buffer pH 9.5 with *B. clausii*, phosphate buffer pH 7.0 with *P. stutzeri* and acetate buffer pH 5.0 with *A. oryzae*). Samples (500 μL) were withdrawn at different time intervals. HCl (0.1 M, 250 μL) and 250 μL of an I_2–KI solution (0.02% I_2 and 0.2% KI) were added and mixed. Deionised water (4.0 mL) was added and mixing was repeated.

Enzyme characterisation.—For the determination of the temperature optimum, the purified enzyme was incubated with a final

concentration of 1% soluble potato starch in 0.1 M borate buffer pH 10.0 (with or without the addition of 5 mM $CaCl_2$) for 15 min at temperatures from 30 to 90 °C. Determination of the temperature stability was performed by incubating the purified enzyme in 50 mM glycine–NaOH buffer pH 9.5 containing 5 mM $CaCl_2$ for 30 min at 30, 40, 50, 55, 60, 70, 80 and 90 °C. Residual activity was determined by incubation of the enzyme with a final concentration of 1% soluble potato starch in 50 mM glycine–NaOH buffer pH 9.5 at 55 °C for 15 min. The pH optimum was determined by incubation of the purified enzyme with a final concentration of 1% soluble potato starch in different buffers at 55 °C for 15 min. The buffers used were 50 mM citrate (pH 4.0–6.0), 50 mM tris-maleate (pH 6.5–8.5), and 50 mM glycine–NaOH (pH 9.0–11.0).

The hydrolysis of different substrates by the purified enzyme was tested with soluble potato starch, amylopectin from corn, dextran, pullulan (all 1%), amylose (0.1%) and α-, β-, and γ-cyclodextrin (10 mM). The substrates were dissolved in 50 mM glycine–NaOH buffer with 5 mM $CaCl_2$ at pH 9.5 and the purified enzyme was added (505 mU mL^{-1}). The various substrates were incubated at 55 °C and samples were withdrawn at different time intervals. The reaction was stopped by boiling for 10 min and the samples were analysed by HPAEC.

The hydrolysis of maltose, maltotriose, and maltotetraose (all 2 mM) and a mixture of maltotetraose to maltodecaose (5 mM) by the purified enzyme were determined. The malto-oligosaccharides were dissolved in 50 mM borate buffer with 5 mM $CaCl_2$ at pH 9.5 and the purified enzyme was added (147 mU mL^{-1}). The substrates were incubated at 55 °C and aliquots were withdrawn at different time intervals. The reaction was stopped by boiling for 10 min and the samples were analysed by HPAEC.

Analysis of hydrolysis products.—Hydrolysis products were detected using HPAEC with pulsed amperometric detection. A CarboPac PA-1 column (Dionex Corporation, Sunnyvale, CA, USA) was used with a gradient of 1.0 M Na–acetate from 0 to 60% over 30 min in 100 mM NaOH and a flow rate of 1.0 mL mL^{-1} on a Dionex DX-300 and DX-500 system. Hydrolysis products were identified by comparison of their retention times with glucose, maltose, maltotriose, maltotetraose, maltopentaose and maltohexaose. Since the retention times of homologous malto-oligosaccharides increase linearly with the DP, malto-oligosaccharides of intermediate DP could be easily identified [40,41]. It was also found that the response for similar molar concentrations of different malto-oligosaccharides decrease with increasing molecular size [40,41].

Amino acid sequencing of the malto-oligosaccharide-forming amylase.—The amylase preparation was subjected to direct amino acid sequencing of the N-terminus as well as to amino acid sequencing after tryptic digestion. Amino acid sequencing was done using a Pulsed Liquid Phase Protein/Peptide Sequencer model 477 and a HPLC On-line PTH-Amino Acid Analyser model 120 A from Applied Biosystems (California) according to manufacturers instructions.

Prior to tryptic digestion, the amylase pool was desalted and concentrated by absorption to a Prosorb (Applied Biosystems) cartridge. The protein absorbed to the membrane was reduced at 60 °C for 1 h in 500 μL 6 M guanidine hydrochloride, 2% MeCN, 0.3% EDTA and 0.2% DTT in 0.5 M Tris–HCl, pH 8.6. It was then carboxymethylated by adding 2.4 mg of iodoacetamide dissolved in 10 μL 0.5 NaOH and stirring for 15 min in the dark. Thereafter the membrane was washed five times with 2% MeCN and sonicated once in 0.1% SDS for 5 min. The carboxymethylated protein was then incubated for 20 min at rte in 0.5% polyvinylpyrrolidone (PVP-40) in 100 mM AcOH. After thorough washing of the membrane with the digestion buffer 1% Triton-X (reduced; Sigma), 5 mM $CaCl_2$, 10% MeCN in 0.1 M Tris–HCl, pH 8.0, the membrane was cut into small pieces and incubated overnight at 37 °C with 10 μg of trypsin (Sigma, sequencing grade from bovine pancreas). Thereafter the mixture was sonicated for 5 min and the supernatant transferred to a fresh tube. Then 100 μL digestion buffer was added to the membrane pieces, the mixture

was sonicated for 30 min and the supernatants were pooled. Then the membrane pieces were washed twice with 200 μL 0.1% TFA in water and the supernatants were pooled. Hereafter sonication was done in 100 μL MeCN with 0.1% TFA for 10 min and the supernatants were pooled. Finally, the membrane pieces were washed with 300 μL 0.1% TFA in water. All the supernatants were pooled and centrifuged for 5 min at 10,000*g* and then applied to a Vydac C18 HPLC column (The Separations Group, California) equilibrated with 0.1% TFA and 10% MeCN in water to purify the peptides using a gradient of 10–60% MeCN over 60 min. Selected peptides were further purified using HPLC on a Develosil C18 column.

The purified peptides were analysed using a Voyager DE (Perspective Biosystems) mass spectrometer applying 1 μL samples co-crystallised with 10 μg μL^{-1} α-cyano-4-hydroxycinnamic acid in 0.1% TFA and 60% MeCN to check their purity and to verify the amino acids sequences obtained.

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