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STARCH METABOLISM IN *PSEUDOMONAS STUTZERI*

I. STUDIES ON MALTOTETRAOSE-FORMING AMYLASE *

JÜRGEN SCHMIDT and MICHAEL JOHN

Lehrstuhl für Biotechnologie, Technische Universität Berlin, Berlin 65 (Germany)

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Summary

The extracellular maltotetraose-forming amylase of *Pseudomonas stutzeri* was purified to homogeneity by a combination of affinity and hydroxyapatite chromatography. Sodium dodecyl sulfate-gel electrophoresis indicated that the oligomeric enzyme contains two different subunits with molecular weights of 48 000 and 58 000. Cross-linking studies using dimethyl suberimidate have demonstrated that the native enzyme consists of dimers. Seven isozymes of the amylase have been identified after polyacrylamide gel electrophoresis and amylose-digestion zymograms. The amylase of *Ps. stutzeri* is known to produce maltotetraose from linear and branched α-glucans by an exomechanism. The relatively high conversion rate of starch (75% hydrolysis), and the hydrolysis of cross-linked blue starch by this amylase indicate that the enzyme can cleave its substrates also by an endomechanism. Further strong evidence for an endomechanism was obtained from the action of the amylase on maltotetraose units which are located within the pullulan molecule. Dextran, pullulan, and maltotetraose are competitive inhibitors. EDTA caused reversible inactivation. Amylase activity could be restored by addition of Ca^{2+}. Heavy metals are inhibitory.

Introduction

The extracellular maltotetraose-forming amylase (1,4-α-D-glucan maltotetrahydrolase, EC 3.2.1.60) is involved in the starch metabolism of *Pseudomonas stutzeri* and has been first described by Robyt and Ackerman [1]. The authors reported that this amylase produces maltotetraose by an exomechanism of

Abbreviation: SDS, sodium dodecyl sulfate.

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substrate encounter. We have evidence that this enzyme is able to degrade α-glucans also by an endomechanism. This communication describes further properties of the *Ps. stutzeri* amylase and summarizes our studies on the structure of this enzyme. A preliminary report of some of these findings has been presented [2].

Materials and Methods

Materials

Pseudomonas stutzeri NRRL B-3389 was kindly supplied by Dr. J.F. Robyt (Iowa State University). Dr. D. Bonse (Miles-Kali-Chemie) supplied a gift of pullulan. Cyclomaltohexaose and cyclomaltoheptaose were obtained from Sigma Chemical Co. Maltotetraose was prepared as described by Robyt and Ackerman [3], with the modification that contaminating oligosaccharides and heavy metals from the ethanol precipitation step [4] were removed by chromatography on Bio-Gel P-2. The freeze-dried crude maltotetraose (2 g) was dissolved in 10 ml water and applied to a Bio-Gel P-2 column (42 × 5 cm) which was eluted with degassed water at 55°C (flow rate 100 ml/h). The collected fractions (5 ml) were assayed for carbohydrate content by the orcinol-sulfuric acid procedure [5]. The fractions containing pure maltotetraose were pooled and lyophilized.

Pullulanase from *Aerobacter aerogenes* (pullulan 6-glucanohydrolase, EC 3.2.1.41) was obtained from Boehringer, Mannheim. α-Amylase from *Bacillus subtilis* (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) was purchased from Serva, Heidelberg. Bio-Gel P-100 (100—200 mesh), Bio-Gel P-2 (minus 400 mesh), and DNA grade Bio-Gel HTP (hydroxyapatite) were purchased from Bio-Rad Laboratories. For optimal column efficiency DNA grade hydroxyapatite was fractionated in warm starting buffer (approx. 60°C) by repeated settling and decanting the fines prior to packing the column. All other reagents used were of the highest grade commercially available.

Enzyme assays

Amylase activity was determined according to the method of Ceska et al. [6] using water-insoluble cross-linked blue starch polymer as substrate. This substrate is commercially available from Pharmacia Fine Chemicals as the Phadebas® amylase test which was used in the course of this investigation. 1 unit amylase activity is defined as that amount of enzyme which will hydrolyze 1 μM glucosidic linkages per min at 37°C and pH 7.0.

Proteolytic activity was assayed by the method of Rinderknecht et al. [7]. Protein concentrations were estimated by the method of Lowry et al. [8], as modified by Schacterle and Pollack [9] with bovine serum albumin as standard.

Purification of amylase

Ps. stutzeri was grown aerobically at 30°C in a Biostat fermentor (Braun, Melsungen, F.R.G.) containing 8.5 l tryptone-yeast extract medium [10] with 1% soluble starch as enzyme inducer. 1 ml polypropyleneglycol 2000 (Baker Chemicals) was used as an antifoaming agent. Other growth conditions were the same as those described previously [10]. Cultivation of the organism

was stopped at early stationary phase, and cells were removed by centrifugation at 14 000 × *g* for 20 min at 0°C. The supernatant was concentrated 2.5-fold by lyophilization. The concentrate was centrifuged at 40 000 × *g* for 10 min at 0°C in order to remove insoluble exocellular polysaccharide which is produced by *Ps. stutzeri* in relatively high yields [11].

All steps in the purification were carried out at 4—6°C unless otherwise indicated. Protein was precipitated by adding solid ammonium sulfate (45% saturation) to the concentrated growth medium.

The maltotetraose-forming amylase was further purified by affinity chromatography as described previously [10] using Sephadex G-100 as affinity adsorbent. After this purification step the fractions containing amylase activity were pooled and dialyzed against 5 mM sodium phosphate (pH 6.8), and then concentrated by ultrafiltration over a PM-10 membrane (Amicon Corp).

Concentrated amylase (2.0 ml) from the previous step was applied to a column (57 × 0.9 cm) of DNA grade hydroxyapatite equilibrated with 5 mM sodium phosphate buffer (pH 6.8). The column was developed as described in Fig. 1. Protein in column effluent was monitored by measuring the transmittance at 280 nm. Peaks of amylase activity were pooled as they eluted from the column to give a fraction A (fractions 67—72) and a fraction B (fractions 81—87). The pooled fractions were concentrated by ultrafiltration over a PM-10 membrane. All concentrated amylase solutions from this final purification step were fully stable for over 3 months when stored at 4°C, and were used in all experiments concerning the properties of the amylase.

Gel electrophoresis

Polyacrylamide gel electrophoresis was performed at pH 8.3 by the method of Davis [12] in 10% acrylamide gel slabs with bromophenol blue as the tracking dye. Electrophoresis was carried out at 40 mA for 3 h at 6°C. Gels were stained for protein with Coomassie brilliant blue G-250 as described recently [13], and for glycoprotein with fuchsin-sulfite [14] and Alcian blue [15].

Bands of amylase activity were detected in the gel by a modification of the technique of Doane [16]. Amylose-polyacrylamide layers (1 mm layer thickness) contained 10% acrylamide, 0.1% *N,N'*-bis-methylene acrylamide, 0.1 M Tris-HCl (pH 7.0) and approx. 1% amylose (Merck). Immediately after electrophoresis the separation gel containing the amylase bands was placed on top of the amylose-gel layer and incubated in a moist chamber for 1 h at 30°C. After staining with iodine and washing with warm water, sites of amylase activity appeared as clear bands on a blue-colored background.

Molecular weight determinations

For estimation of subunit molecular weight, sodium dodecyl sulfate-gel electrophoresis was carried out in 10% polyacrylamide slabs by the method of Laemmli [13]. Electrophoresis was performed at 20°C with a current of 30 mA until the dye marker (bromophenol blue) reached the bottom of the gel (about 3 h). Gels were stained with Coomassie brilliant blue as described recently [13]. Mobilities of protein bands were calculated by the method of Weber and Osborn [17] and were plotted against the known molecular weights of the marker proteins expressed on a semi-logarithmic scale.

Cross-linking of the amylase with dimethyl suberimidate (Eastman, Rochester) was performed by the method of Carpenter and Harrington [18]. SDS-gel electrophoresis of the cross-linked enzyme was carried out as previously described [19], and molecular weights were estimated by this procedure employing cross-linked ovalbumin [18] with known molecular weights (43 000—258 000) as marker proteins.

Gel chromatography of reduced and alkylated proteins in 6 M guanidine-HCl was used for the further estimation of polypeptide chain molecular weight. The procedure was carried out with the use of a Sepharose CL-6B column (78 × 1.5 cm) as described by Mann and Fish [20].

Enzymic digests and gel chromatography of reaction products

Soluble starch was incubated with *Ps. stutzeri* amylase as indicated in the legend to Fig. 4. The products in the digests were determined by gel chromatographic analysis of 90-μl aliquots. Mono- and oligosaccharides were separated on a column (200 × 1.5 cm) of Bio-Gel P-2 (minus 400 mesh) at 65°C as described previously [21]. Carbohydrates in the column effluent were detected and quantitatively measured by the orcinol-sulfuric acid method using an autoanalyzer.

Hydrolysis of pullulan was studied for the maltotetraose-forming amylase and for the α-amylase from *B. subtilis*. The enzymes were added to the incubation mixture so that there was 1 amylase unit for each milligram of substrate. The reaction was carried out at 37°C and pH 7.0 for 18 h. The products of pullulan degradation were separated on a column (34 × 2.5 cm) of Bio-Gel P-100 (100-200 mesh) using water as eluant. The column effluent was monitored continuously for carbohydrates by a differential refractometer. Pullulanolysis of the amylase-treated pullulan was performed at pH 5.0 and 37°C until no further increase in reducing power was observed. Reducing sugars were determined by the dinitrosalicylic acid procedure [22]. The products were separated by column chromatography on Bio-Gel P-2 [21].

Results

Purification of enzyme

The maltotetraose-forming myalase of *Ps. stutzeri* was purified to homogeneity on Sephadex G-100 as an affinity adsorbent. The enzyme binds to dextran gel because dextran is a competitive inhibitor of the amylase [10]. Displacement of the bound amylase was carried out using 1% soluble starch in phosphate buffer (pH 7.0) as a highly specific eluant.

The major impurity in the amylase eluate from the affinity column is starch, which was removed by subsequent chromatography of the amylase on hydroxyapatite. Furthermore, this procedure separated the enzyme in two active fractions, both having a high product specificity for the formation of maltotetraose (Fig. 1). This high resolution could only be achieved by the use of small particle-sized hydroxyapatite (DNA grade) in a relatively long column. The column eluate fractions were pooled according to the hatched areas in Fig. 1, to give fractions A and B. Fraction A was eluted with 57 mM sodium phosphate, whereas 66 mM phosphate buffer (pH 6.8) was required to elute

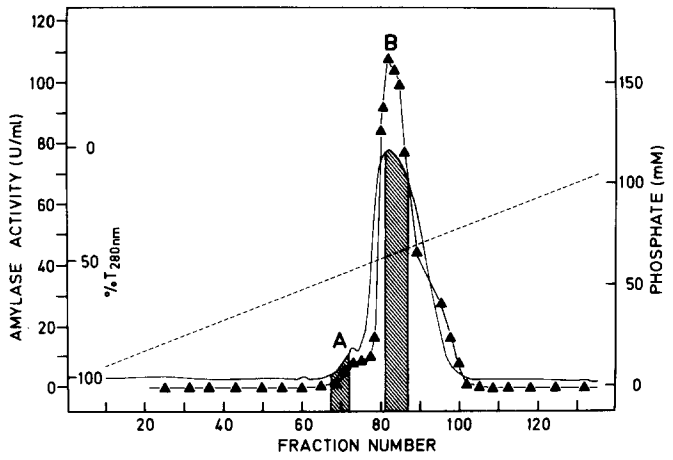

Fig. 1. Chromatography of amylase on DNA grade hydroxyapatite. The sample (2.0 ml; 17.1 mg protein per ml) was applied to the column (57 × 0.9 cm) in 5 mM sodium phosphate buffer (pH 6.8). The enzyme was eluted with a linear gradient (150 ml in each chamber) of sodium phosphate (pH 6.8) from 5 mM to 150 mM (flow rate, 8 ml/h; 1.5-ml fractions). The fractions of the hatched peaks A and B were pooled separately. ———, % transmittance at 280 nm; - - - - - -, sodium phosphate gradient; ▲———▲, amylase activity.

fraction B. No other protein peaks could be observed, indicating that the enzyme was pure. The homogeneity of the amylase preparation from the hydroxyapatite step was further evaluated by polyacrylamide and SDS-gel electrophoresis as shown later. No proteolytic activity was detected in the purified enzyme preparation or in the crude ammonium sulfate concentrate of the culture medium using Remazolbrilliant Blue-hide powder as substrate [7]. The specific activity of the amylase obtained after hydroxyapatite chromatography was 209 units per mg protein, corresponding to a 246-fold purification over the initial cell-free culture medium.

Molecular weight and number of subunits

Discontinuous SDS-gel electrophoresis of the two amylolytic active fractions from hydroxyapatite chromatography revealed that the major fraction B consists of two different subunits in roughly equal proportions (Fig. 2, gel C). The upper protein band was designated subunit A and the lower subunit B. The minor fraction A is composed of only 1 subunit (Fig. 2, gel D). As can be seen in Fig. 2, the single polypeptide chain of fraction A (gel D) migrated at the same rate as subunit B (gel C), which indicates that both subunits are of the same size. The molecular weight of subunit A estimated by SDS-gel electrophoresis was 58 000, and subunit B had a molecular weight of 48 000. These values agreed well with the molecular weight estimation of the reduced and alkylated amylase by gel chromatography in the presence of 6 M guanidine-HCl. By this technique the two different subunits emerged as a single peak from the gel column, and a molecular weight of 55 000 was estimated from its position of elution.

These results and the results concerning the isozymic forms of the enzyme described below indicate that the *Ps. stutzeri* amylase appears to be an oligomeric protein composed of two kinds of subunit.

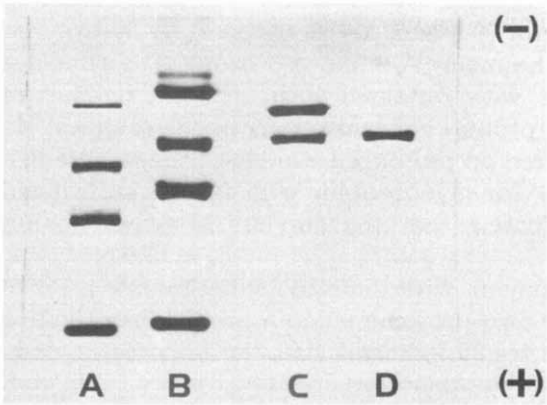

Fig. 2. Discontinuous SDS-gel electrophoresis of pooled amylase fractions A and B obtained after hydroxyapatite chromatography. The following standard proteins were used: Gel A (from top to bottom), catalase (molecular weight 58 000), aldolase (40 000), chymotrypsinogen A (25 700), cytochrome *c* (11 700); gel B, bovine serum albumin (68 000), ovalbumin (43 000), lactate dehydrogenase (36 000), myoglobin (17 200). Gel C shows the protein bands of fraction B and gel D illustrates the polypeptide chain of fraction A (See Fig. 1). Migration direction was from the top.

Isozymes

Polyacrylamide gel electrophoresis of the combined amylase fractions A and B (Fig. 1) showed seven protein bands (Fig. 3A). Before staining the gel for protein with Coomassie blue, a zymogram was prepared by placing the separation gel on an amylose-containing polyacrylamide gel. Subsequent staining with iodine revealed that all seven protein bands possessed amylase activity (Fig. 3B). The isozyme band removing farthest towards the anode is designated as A-1 and the slowest moving band A-7. As can be further deduced from the

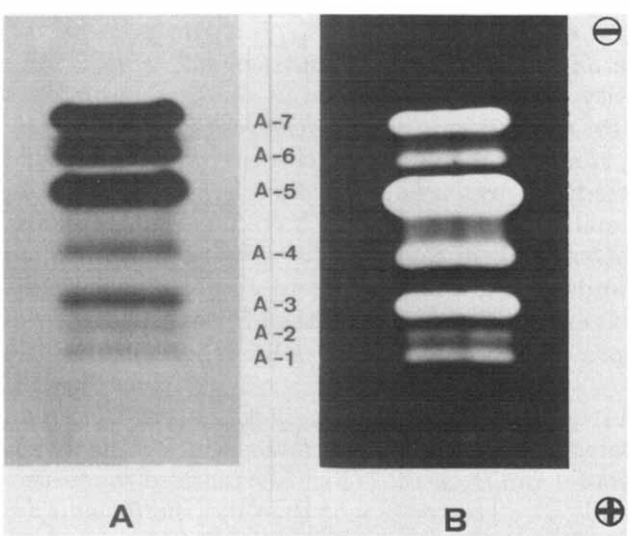

Fig. 3. Isozyme pattern of *Ps. stutzeri* amylase. The purified enzyme was subjected to electrophoresis in 10% polyacrylamide gel. Gel A was stained for protein with Coomassie blue. The zymogram (B) shows the pattern of the amylase isozymes on an amylose-containing polyacrylamide gel plate stained with iodine.

isozyme patterns in Fig. 3, the minor A-3 and A-4 protein bands hydrolyzed the amylose much faster than did the major A-6 and A-7 bands. The same distribution and number of isozymes were obtained with different batches of purified enzyme and with different protein concentrations in the samples. No change in the isozyme pattern occurred on prolonged storage of the samples.

Specific staining of the isozymes for glycoprotein with fuchsin-sulfite and Alcian blue was unsuccessful, indicating that the amylase isozymes do not contain carbohydrates.

Cross-linking of the oligomeric enzyme with dimethyl suberimidate prior to dodecyl sulfate electrophoresis gave two protomers and a protein band with a molecular weight of 120 000. This result indicates that the oligomer is composed of dimers as has been suggested by other workers [1].

Mode of action

The hydrolysis of starch and pullulan was studied for the purified amylase of *Ps. stutzeri*, and the degradation products were analyzed by polyacrylamide gel chromatography. Characteristic action patterns were obtained for the enzyme after 2 and 24 h of incubation with soluble starch (Fig. 4). During the first two hours of hydrolysis the amylase degraded starch exclusively to maltotetraose (Fig. 4A). As shown in the inset of Fig. 4A the conversion of starch into maltotetraose (determined by gel chromatography) was approx. 55% after 2 h incubation. The formation of maltotetraose as single degradation product during the initial stage of hydrolysis indicates an exomechanism of substrate encounter as was suggested by Robyt and Ackerman [1].

At relatively high enzyme concentrations and prolonged incubation (24 h), however, dextrins and maltotetraose were slowly degraded to maltotriose, maltose, and glucose (Fig. 4B). Under these conditions starch was hydrolyzed to the extent of about 75%. This relatively high conversion rate cannot be explained merely by an exomechanism. Moreover, cross-linked blue starch, which is a specific substrate for endo-amylases [23,24], is hydrolyzed by the maltotetraose-forming amylase and was used in the course of this work for the determination of amylase activity.

Further evidence that the *Ps. stutzeri* amylase can cleave α-glucans also by endo-attack was obtained by studying the action of the enzyme on pullulan. Pullulan is a linear α-glucan produced by *Pullularia pullulans*, in which maltotriose and small amounts of maltotetraose units are linked by 1,6-glycosidic bonds [25,26]. Catley and Whelan [27] showed that the maltotetraose units are located within the polysaccharide and are susceptible to endo-amylase attack [28]. The pullulan we used in these experiments contained 5% maltotetraose as evidenced by gel chromatography on Bio-Gel P-2 after exhaustive hydrolysis by pullulanase.

The degradation of pullulan by the amylase of *Ps. stutzeri* and the endo-amylase of *B. subtilis* is illustrated in Fig. 5. As can be deduced from the different elution profiles, the α-amylase from *B. subtilis* (Fig. 5B) reduced the molecular weight of the polysaccharide to a greater extent than did the *Ps. stutzeri* amylase (Fig. 5A). The degradation products deriving from the action of *Ps. stutzeri* amylase on pullulan were excluded from a Bio-Gel P-2 column, indicating that they have molecular weights higher than 3000. That is why these

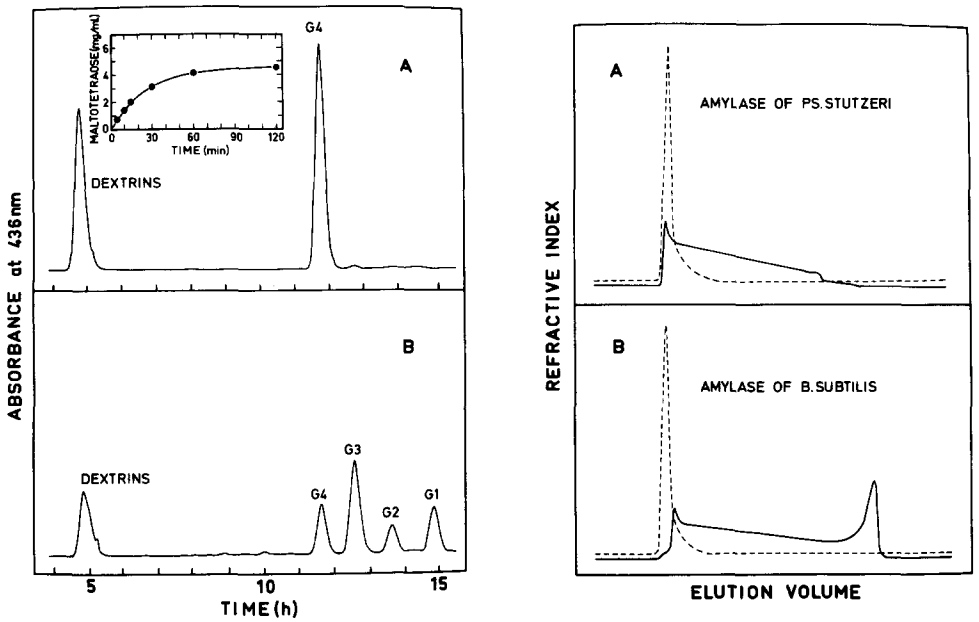

Fig. 4. Action of amylase from *Ps. stutzeri* on soluble starch and gel chromatography of the degradation products. (A) Starch (8 mg per ml) was incubated with the enzyme (120 mU/ml) in phosphate buffer (pH 7.0) at 37°C for 120 min. (B) Starch was incubated with the enzyme (3.7 U/ml) at 37°C for 24 h. Gel chromatographic analysis of the hydrolyzate was carried out on a Bio-Gel P-2 column (200 X 1.5 cm). The inset shows the formation of maltotetraose during the first hours of incubation. Peaks G1, G2, G3, G4 denote glucose, maltose, maltotriose, maltotetraose.

Fig. 5. Hydrolysis of pullulan by two different amylases and gel chromatography of the degradation products on Bio-Gel P-100. A sample (8 mg) was applied to the column (34 X 2.5 cm) and eluted with degassed water at 45°C (flow rate, 25 ml/h). Column eluate was monitored by means of a differential refractometer. The broken line shows the elution profile of pullulan prior to degradation with amylase. The solid line indicates the elution pattern of pullulan after treatment with amylase. (A) Digest with amylase of *Ps. stutzeri*, (B) digest with amylase of *B. subtilis*.

degradation products could not arise from the action of a pullulanase. The pullulan which was modified by the *Ps. stutzeri* amylase was incubated with pullulanase and the resulting oligosaccharides were analyzed by gel chromatography. As shown in Fig. 6, treatment with pullulanase yielded maltotetraose, a tetrasaccharide which was probably 6^3-α-glucosylmaltotriose, maltotriose, and maltose. The traces of a pentasaccharide may represent maltosylmaltotriose which had not been hydrolyzed by pullulanase. The small amount of maltotetraose in the hydrolyzate indicates that not all of the maltotetraose units were cleaved by amylase action. The presence of maltose and glucosylmaltotriose in this digest shows that the amylase of *Ps. stutzeri* acts on pullulan by cleaving internally located maltotetraose units, as illustrated in the proposed scheme of action (Fig. 7).

Kinetic properties

Dextran inhibits the amylase competitively [10]. A concentration of 5 mg dextran per ml digest caused approx. 50% inhibition of amylase activity. Pullulan is a more effective competitive inhibitor for the enzyme with a K_i of

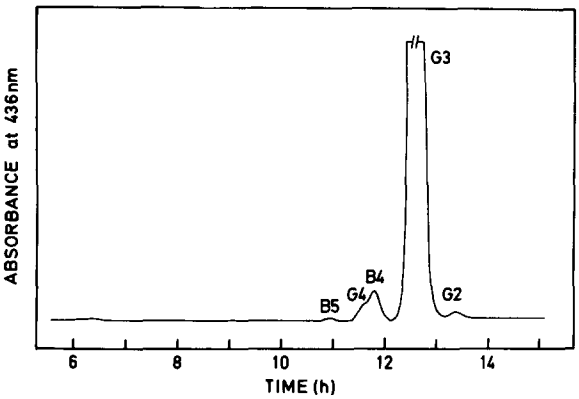

Fig. 6. Gel chromatographic separation of oligosaccharides on Bio-Gel P-2. Oligosaccharides were obtained after degradation of pullulan with amylase of *Ps. stutzeri* and subsequent treatment with pullulanase. Peaks G2, G3, G4 denote maltose, maltotriose, maltotetraose; B4, tetrasaccharide; B5 pentasaccharide.

0.6 mg per ml, as determined by the method of Dixon [29]. Maltotetraose shows also competitive inhibition with a K_i of 3.0 mM when cross-linked blue starch was the substrate.

Activation of amylase activity was observed, when incubating the enzyme with cross-linked blue starch in the presence of varying amounts of cyclomaltoheptaose (Fig. 8). The reason for this has not been established, but obviously cyclomaltoheptaose increases the susceptibility of this particular substrate. This effect has not been found with soluble starch as substrate. Cyclomaltohexaose had no effect on amylase activity.

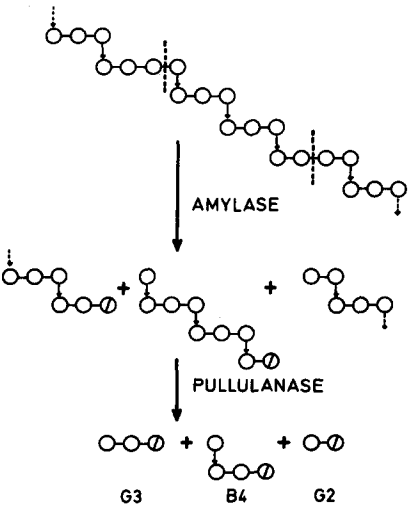

Fig. 7. Proposed scheme of action of *Ps. stutzeri* amylase on the maltotetraose units within the pullulan molecule and subsequent pullulanolysis. 1,6-α-Linkages are represented by ↓; 1,4-α-linkages by —. Non-reducing glucose units are shown as ○ and reducing units as ∅. Linkages susceptible to amylase attack are represented by ┼. G2, maltose, G3 maltotriose; B4, 6^3-α-glucosylmaltotriose.

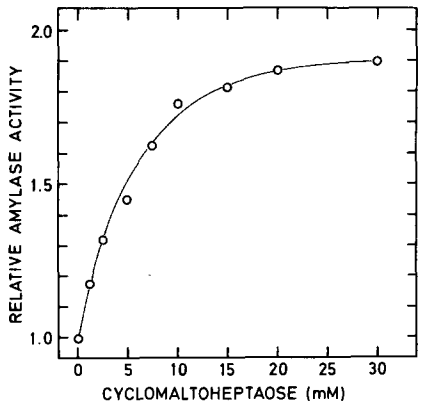

Fig. 8. Effect of cyclomaltoheptaose on amylase activity using cross-linked blue starch as substrate.

The basal activity was greatly stimulated in the presence of Ca^{2+}. Addition of 10 mM EDTA to the incubation mixture resulted in a reversible inactivation of the enzyme. Amylase activity could be restored by addition of 15 mM $CaCl_2 \cdot 4 H_2O$, suggesting that the enzyme is a metalloprotein. Incubation of the amylase with 50 mM iodoacetamide resulted in 57% inactivation after 4 h. Subsequent treatment with 2-mercaptoethanol or dithiothreitol did not reverse the inhibition. The enzyme was strongly inhibited by 1 mM Hg^{2+}, Cu^{2+}, and Zn^{2+}.

Discussion

The preparation of homogeneous amylase in sufficient quantities has been facilitated by the use of dextran gel as an efficient affinity adsorbent. The binding of the maltotetraose-forming amylase to Sephadex G-100 is caused by a specific interaction of the enzyme with dextran which is a competitive inhibitor of the amylase. The capacity for binding amylase is high and nonspecific protein binding was not detected. This property of the *Ps. stutzeri* amylase seems to be unique, since α-amylases from other sources are only slightly retained on dextran gel columns [10,30]. On the other hand, typical exo-amylases e.g., β-amylase from sweet potatoes and amyloglucosidase from *Aspergillus niger*, showed no such retention on a bed of dextran gel (Schmidt, J., unpublished data).

Dissociation of the purified amylase in SDS and subsequent gel electrophoresis revealed that the enzyme consists of two kinds of subunits with molecular weights of 48 000 and 58 000, respectively. These values were confirmed by gel chromatography of the reduced amylase in the presence of 6 M guanidine-HCl. Using gel chromatography on Bio-Gel P-150, Robyt and Ackerman [1] found only one subunit in the native enzyme with a molecular weight of 12 500. Attempts were made to estimate the molecular weight of the native enzyme by chromatography on Bio-Gel P-150 and Sepharose 6B. In both cases it turned out that the amylase was markedly retarded on polyacrylamide gel and to a much greater extent on agarose gel under normal chromatographic conditions (Witt, W., unpublished data). Retardation of human amylases on columns of Bio-Gel P-150 has been already observed by other workers [31]. This finding may possibly explain the rather low subunit molecular weight of the *Ps. stutzeri* amylase reported by Robyt and Ackerman [1].

The same authors have reported the presence of at least two isozymic forms of the amylase. We have detected seven isozymes of the amylase by means of polyacrylamide gel electrophoresis and amylose-digestion zymograms. It is rather unlikely that the isozymes are artifacts due to proteolysis, since no minor protein bands in addition to the two subunit bands could be observed after discontinuous dodecyl sulfate-gel electrophoresis. Moreover, no proteolytic activity could be detected in the amylase-containing fractions during the purification procedure.

Robyt and Ackerman [1] reported that the enzyme is an oligomeric protein in which the protomers are arranged mainly as dimers. These dimers associate to give active oligomers [1]. The predominance of dimers in the quarternary structure of the amylase was confirmed by us in cross-linking studies of the native enzyme using dimethyl suberimidate. The existence of seven isozymes

could be explained by the assumption that the native enzyme is composed of three dimers to give a hexameric structure for the protein. According to this assumption the two different subunits, designated as A and B, may associate to form seven types of hexamer of the following compositions: A_6, BA_5, B_2A_4, B_3A_3, B_4A_2, B_5A, and B_6. This hypothesis is supported by the finding that the minor fraction A from the hydroxyapatite column (Fig. 1) contained only B subunits (Fig. 2, gel D), whereas the main peak (Fig. 1, fraction B) contained the hybrid forms which are composed of A and B subunits (Fig. 2, gel C). Nevertheless, further evidence has to be presented to confirm this hypothesis of amylase isozyme structure.

At early stage of hydrolysis the amylase of *Ps. stutzeri* degrades starch to form maltotetraose in high yields. The corresponding sugar alcohol of maltotetraose can be used as a substrate for the specific determination of β-amylase activity in the presence of α-amylase in germinated barley (John et al. [4,32]). The exclusive formation of maltotetraose suggests an exomechanism of substrate encounter. Other properties, however, such as the relatively high conversion rate of soluble starch (75% hydrolysis) and the hydrolysis of cross-linked blue starch by this enzyme indicate that the amylase of *Ps. stutzeri* can cleave α-glucans also by an endomechanism. Further strong evidence for an endomechanism was obtained from the action of the amylase on maltotetraose units which are located within the pullulan molecule. Nevertheless, it has not yet been possible to establish how the amylase can have both mechanisms of substrate encounter. As shown in this paper (Fig. 3), the amylase isozymes showed different catalytic properties when acting on amylose, and future investigations of the mode of action of the single isozymes may possibly provide more insights into the enzyme mechanism.

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