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# STARCH METABOLISM IN *PSEUDOMONAS STUTZERI*

## II. PURIFICATION AND PROPERTIES OF A DEXTRIN GLYCOSYLTRANSFERASE (D-ENZYME) AND AMYLOMALTASE *

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## Summary

Amylomaltase and disproportionating enzyme (D-enzyme) were purified to homogeneity from cell-free extracts of *Pseudomonas stutzeri* using a six-step procedure. The presence of both glycosyltransferases in the same organism has not been reported before. Molecular weight determination by gel chromatography gave a value of 74 000 for the amylomaltase and 115 000 for the D-enzyme. Two subunits of different molecular weight were found in each enzyme as proved by sodium dodecyl sulfate-gel electrophoresis. The optimum pH of amylomaltase and D-enzyme activity is 7.6—7.7. Action of both glycosyltransferases on different maltodextrins showed that amylomaltase is most active with maltotetraose, and the $K_m$ value for this substrate is 7.1 mM. D-Enzyme catalyzed glucose release from maltose ($K_m$ = 8.3 mM) at a higher rate than from maltotriose and maltotetraose. With maltotriose as initial substrate, D-enzyme forms glucose, maltopentaose, maltoheptaose, maltononaose, maltoundecaose as major products. Amylomaltase acts on maltotriose, maltotetraose, and maltopentaose to form a series of homologous 1,4-α-glucans. No essential chain-lengthening reaction occurred with maltohexaose.

## Introduction

The utilization of starch by *Pseudomonas stutzeri* involves the extracellular degradation of the polysaccharide to maltodextrins which are further converted intracellularly into glucose and glucose-1-phosphate. A pathway for the metabolism of maltodextrins in *Ps. stutzeri* has been proposed recently [1]. The enzymes involved in this pathway are maltodextrin permease, amylomaltase

* This paper is part of a thesis of J. Schmidt to Fachbereich Biologie der Freien Universität Berlin. Abbreviation: SDS, sodium dodecyl sulfate.

(1,4-α-D-glucan:1,4-α-D-glucan 4-α-glycosyltransferase, EC 2.4.1.25), and maltodextrin phosphorylase (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1). In a preliminary report [2] we showed that *Ps. stutzeri* contains in addition to amylomaltase a further glycosyltransferase which resembles the plant D-enzyme (disproportionating enzyme or dextrin glycosyltransferase; 1,4-α-D-glucan:1,4-α-D-glucan 4-α-glycosyltransferase, EC 2.4.1.25), first described by Peat et al. [3]. The D-enzyme of *Ps. stutzeri* is clearly different from amylomaltase as evidenced by action patterns and other properties. The occurrence of both glycosyltransferases in the same organism has not been reported before. In this paper we describe the purification and characterization of amylomaltase and D-enzyme from *Ps. stutzeri*, and we propose a scheme for the metabolism of starch in this microorganism.

## Materials and Methods

### *Materials*

*Pseudomonas stutzeri* NRRL B-3389 was a gift of Dr. J.F. Robyt (Iowa State University). β-Amylase from sweet potatoes, pullulanase from *Aerobacter aerogenes*, hexokinase, glucose-6-phosphate dehydrogenase, and phosphoglucomutase were obtained from Boehringer, Mannheim. α-Amylase from *Bacillus subtilis* was a gift of Röhm and Haas, Darmstadt. Sepharose 6B, DEAE-Sephadex A-50, and Sephadex G-200 were purchased from Pharmacia Fine Chemicals. Bio-Gel A-1.5 (200—400 mesh), Bio-Gel P-150 (100—200 mesh), Bio-Gel P-2 (minus 400 mesh), and DNA grade Bio-Gel HTP (hydroxyapatite) were supplied by Bio-Rad Laboratories. In order to achieve optimal chromatographic resolution, Bio-Gel HTP (DNA grade) was fractionated in warm starting buffer (approx. 60°C) by repeated settling and decanting the fines prior to pouring the column.

Partially purified amylomaltase from *Escherichia coli* ML 30 was prepared by chromatography of the crude cell extract on a Bio-Gel A-1.5 column (68 × 2.5 cm) equilibrated with 0.1 M phosphate (pH 7.0). The final enzyme preparation had a specific activity of approx. 0.3 units per mg protein and was free of maltodextrin phosphorylase activity.

Commercial maltose (Merck) was freed of contaminating mono- and oligosaccharides by gel chromatography on a column (200 × 1.5 cm) of Bio-Gel P-2. Maltotriose was prepared by exhaustive hydrolysis of pullulan (Serva, Heidelberg) with pullulanase and was purified by chromatography on Bio-Gel P-2. Maltotetraose was prepared as described in the preceding paper [4]. Maltopentaose was obtained by digestion of amylose (Merck) with α-amylase from *B. subtilis* and was isolated by gel chromatography. Maltohexaose was a generous gift of Dr. B.S. Enevoldsen (Carlsberg Research Center). A mixture of maltodextrins was prepared by incubation of maltotetraose with amylomaltase from *E. coli*. The reaction mixture was freed of glucose, maltose, and maltotriose by gel chromatography on Bio-Gel P-2.

All other chemicals were of highest grade commercially available.

### *Enzyme assays*

Assay mixtures for the determination of amylomaltase and D-enzyme activ-

ity contained 15 mM maltotetraose and 0.1 M phosphate (pH 7.6). Enzyme was added to initiate the reaction and the mixtures were incubated at 30°C for 10—30 min. Reaction was stopped by heating (90°C, 5 min). The glucose released was estimated by the hexokinase method [5]. 1 unit of glycosyltransferase activity will release 1 $\mu$M glucose per min under these assay conditions. An alternate assay used to determine the properties of the purified D-enzyme employed 15 mM maltose as substrate.

Maltodextrin phosphorylase was assayed by measuring the release of glucose 1-phosphate from a maltodextrin mixture. The liberated glucose 1-phosphate was determined by the method of Munch-Petersen and Kalckar [6]. Amylase and protease activity was determined as previously reported [4].

*Analytical methods*

Action patterns were obtained for amylomaltase and D-enzyme after incubation of the purified enzymes with different maltodextrins and subsequent gel chromatography of the reaction products. The substrates (20 mg per ml) were dissolved in 0.1 M phosphate buffer (pH 7.6). Purified enzyme (2.5 mU per mg substrate) from Step 6 was added to initiate the reaction, and the mixtures were incubated at 30°C with toluene added as preservative. After 16 h the same amount of purified enzyme was added to the reaction mixtures, and the solutions were incubated for further 14 h. The reaction was stopped by heating (90°C, 3 min), and the solutions were centrifuged. The products were determined by gel chromatographic analysis of 90-$\mu$l aliquots of the reaction mixtures. Mono- and oligosaccharides were separated on a column (200 × 1.5 cm) of Bio-Gel P-2 as described previously [7].

In order to prove that the reaction products are members of a homologous series, the negative logarithm of the distribution coefficient of the oligosaccharides was plotted against their molecular weight as described by Hjertén [8]. Gas-liquid chromatography of carbohydrates was carried out as by Sweeley and coworkers [9] using a stainless-steel column (300 × 0.4 cm innerdiameter) packed with 3% OV-17 on HP Chromosorb G AW-DMCS (80—100 mesh) [10].

Protein was determined by the method of Lowry et al. [11], as modified by Schacterle and Pollack [12]. Column effluents were monitored continuously for proteins by measuring the transmittance at 280 nm.

*Molecular weight determinations*

Molecular weights of the purified glycosyltransferases were estimated with the use of a calibrated Sephadex G-200 column (81 × 25 cm) which was eluted with 0.1 M phosphate buffer (pH 7.0).

For estimation of subunit molecular weight, SDS-gel electrophoresis was performed in 12.5% acrylamide gel slabs by the method of Laemmli [13], as detailed in the preceding paper [4].

*Purification of amylomaltase and D-enzyme*

Large-scale growth of *Ps. stutzeri* was accomplished as described [4]. Cells were harvested in early stationary phase by centrifugation (14 000 × *g*, 20 min, 0°C) and washed twice in ice-cold 0.1 M phosphate buffer (pH 7.0). Cells were stored as a frozen paste at —20°C until use.

*Step 1: cell breakage.* Approx. 60 g (wet weight) of cells were thawed at

room temperature and were suspended in 300 ml cold 0.1 M phosphate buffer (pH 7.0). The cells were disrupted by sonication with a Branson sonifier (Model S-125, Branson Sonic Power, Danbury, Conn., U.S.A.) for a total of 2 min. The temperature was maintained at 4—8°C by cooling with an ice bath. The disrupted cell suspension was then centrifuged at 48 000 × *g* for 40 min. All subsequent procedures were performed at 0—6°C.

*Step 2: ammonium sulfate precipitation.* The supernatant solution (approx. 300 ml) was diluted with the same volume of ice-cold 0.1 M phosphate buffer (pH 7.0). Solid ammonium sulfate was slowly added to the stirred solution to achieve 70% saturation. The precipitated protein was collected by centrifugation at 48 000 × *g* for 5 min. An appropriate amount of the resulting precipitate (280 mg protein) was dissolved in a minimum volume (7.0 ml) of 0.1 M phosphate buffer (pH 7.0).

*Step 3: chromatography on Sepharose 6B.* The solution from Step 2 was applied to a Sepharose 6B column (80 × 2.5 cm) previously equilibrated with 0.1 M phosphate buffer (pH 7.0). The proteins were eluted with the same buffer (flow rate 12 ml/h) and collected in 3.0-ml fractions. The fractions containing the glycosyltransferase activity (fractions 75—95) were combined and concentrated by ammonium sulfate precipitation (70% saturation). The precipitate was dissolved in 0.1 M Tris-HCl (pH 7.6) and dialyzed against the same buffer. The dialyzed fraction was then concentrated to approx. 4 ml by ultrafiltration over a PM 10 membrane (Amicon Corp).

*Step 4: DEAE-Sephadex A-50 chromatography.* The dialyzed fraction was applied to a column (20 × 2.5 cm) of DEAE-Sephadex A-50 equilibrated in 0.1 M Tris-HCl (pH 7.6). The column was first eluted with a 600-ml linear sodium chloride gradient (0—0.5 M NaCl in 0.1 M Tris-HCl, pH 7.6). The column was then washed with 0.1 M Tris-HCl (pH 7.6) containing 0.5 M NaCl until the transferase peak had been eluted. Fractions (4.3 ml) were collected at a flow rate of 20 ml per h. The fractions containing transferase activity were pooled and dialyzed against 70 mM sodium phosphate (pH 7.0). The dialyzed fraction was concentrated by ultrafiltration (PM 10 membrane).

*Step 5: chromatography on Bio-Gel P-150.* The concentrated enzyme solution (2.5 ml) from Step 4 was applied to a column (110 × 2.5 cm) of Bio-Gel P-150 previously equilibrated with 70 mM sodium phosphate (pH 7.0). The column was eluted with the same buffer at a flow rate of 8 ml per h. The fractions (3.0 ml) constituting peak A (fractions 57—61) and peak B (fractions 68—73) were separately pooled. The pooled fractions were dialyzed against 5 mM sodium phosphate (pH 6.8), and then concentrated by ultrafiltration.

*Step 6: hydroxylapatite chromatography.* The concentrated enzyme solutions from Step 5 were applied to column (60 × 0.9 cm) of hydroxyapatite previously equilibrated with 5 mM sodium phosphate buffer (pH 6.8). The column was developed with a 300-ml linear sodium phosphate gradient (pH 6.8) from 5 to 200 mM. Elution was performed at a flow rate of 8 ml per h with a fraction size of 1.5 ml. The fractions containing activity were combined and concentrated by ultrafiltration (Minicon B 15, Amicon Corp). The protein of the final samples was precipitated by the addition of ammonium sulfate. The enzyme preparations obtained from this step were used for all studies on the characterization of the glycosyltransferases.

## Results

*Induction of transferase*

When growing *Pseudomonas stutzeri* in a synthetic glycerol medium, the addition of maltotetraose induces the formation of glycosyltransferase in this microorganism (Fig. 1). As also shown in Fig. 1, the enzyme formation is a linear function of the increase in bacterial mass. The same increase in activity was also observed with maltose as inducer. Similar kinetics have been described for the induction of amylomaltase in *E. coli* by maltose [14]. The experiment illustrated in Fig. 1 also revealed that glucose at a concentration of 2 mM prevented temporarily the synthesis of glycosyltransferase when added together with 2 mM maltotetraose to uninduced cells of *Ps. stutzeri*, growing on glycerol. During these studies no intracellular amylase activity could be detected as was already reported by other workers [15].

*Enzyme purification*

D-Enzyme has been separated from amylomaltase of *Ps. stutzeri* by a six-step purification of the cell extract. A summary of the purification is given in Table I. The purification of the enzymes was complicated by an exocellular polysaccharide which is elaborated by *Ps. stutzeri* [16]. Although the cells were washed intensively, it was not possible to remove the polymer completely from the cell surface. Chromatography on Sepharose 6B (Fig. 2) yielded a partial separation of the transferase peak from the polymer. Extracellular amylase adhered to the polysaccharide and passed together with this slimy high-molecular-weight material through the Sepharose column. DEAE-Sephadex chroma-

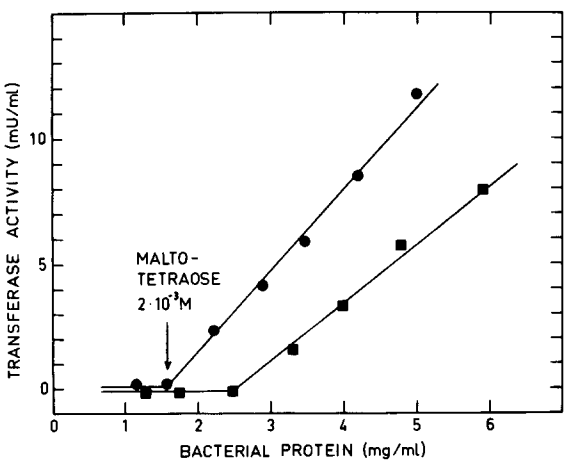


Fig. 1. Induction of glycosyltransferase by maltotetraose and effect of glucose on enzyme synthesis. At the point indicated 2 mM maltotetraose (●——●) was added to a culture of *Ps. stutzeri* growing exponentially in a synthetic medium containing 0.5% glycerol as sole source of carbon. In another experiment 2 mM maltotetraose and 2 mM glucose (■——■) were added simultaneously to exponentially growing cells at the point indicated by the arrow. Aliquots were removed every 60 min and centrifuged. Cells were washed twice and disrupted by sonication. Enzyme assays and protein determinations were performed as described in Materials and Methods.

TABLE I

PURIFICATION OF GLYCOSYLTRANSFERASES FROM *PSEUDOMONAS STUTZERI*

| Step | | Activity (mU/ml) | Protein (mg per ml) | Specific activity (mU/mg protein) | Purification factor |
|---|---|---|---|---|---|
| 1 | Crude extract | 57 | 14.5 | 4 | 1 |
| 2 | Precipitation with ammonium sulfate (70% saturation) | 179 | 28.3 | 6.3 | 1.6 |
| 3 | Chromatography on Sepharose 6B | 91 | 2.27 | 40 | 10 |
| 4 | Chromatography on DEAE-Sephadex A-50 | 62 | 1.29 | 48 | 12 |
| 5 | Chromatography on Bio-Gel P-150 | | | | |
| | (a) D-enzyme | 208 | 1.1 | 189 | 47 |
| | (b) amylomaltase | 201 | 0.73 | 275 | 69 |
| 6 | Hydroxylapatite chromatography | | | | |
| | (a) D-enzyme | 82 | 0.21 | 391 | 98 |
| | (b) amylomaltase | 99 | 0.1 | 990 | 248 |

tography removed the final traces of the polysaccharide and achieved a complete separation of the maltodextrin phosphorylase from amylomaltase and D-enzyme.

Gel chromatography on Bio-Gel P-150 (Fig. 3) separated the D-enzyme (peak A) from amylomaltase (peak B) according to their different molecular

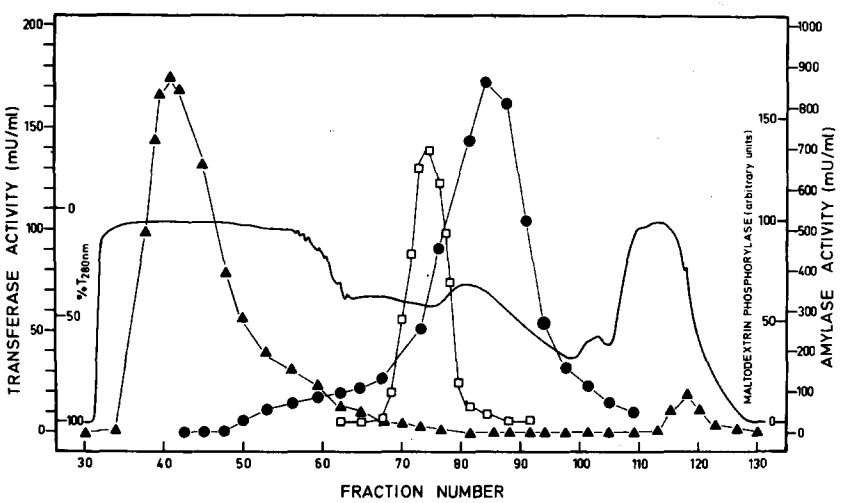


Fig. 2. Chromatography of the 70% ammonium sulfate fraction from Step 2 on Sepharose 6B. The sample (7.0 ml) was applied to the column (80 × 2.5 cm) in 0.1 M phosphate buffer (pH 7.0). The column was eluted with the same buffer at a flow rate of 12 ml per h. 3.0-ml fractions were collected. ———, % transmittance at 280 nm; ●———●, glycosyltransferase activity; ▲———▲, amylase activity; □———□, maltodextrin phosphorylase activity.

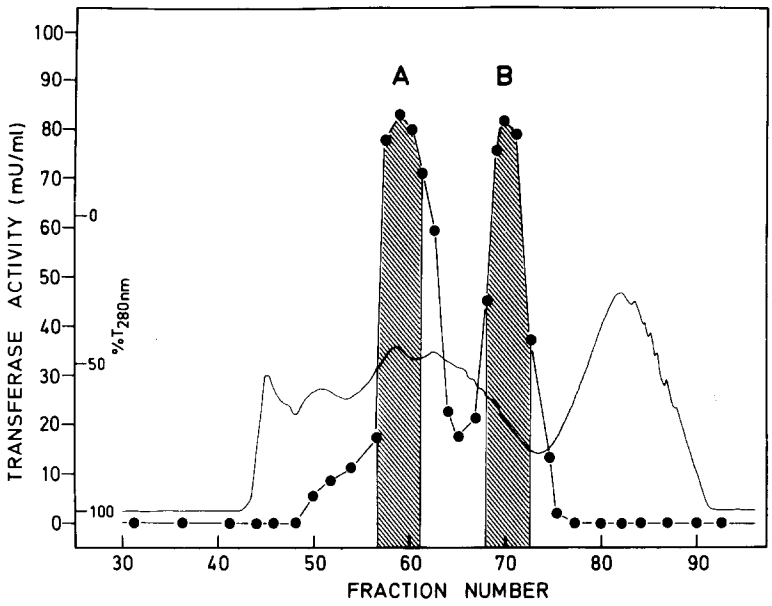


Fig. 3. Separation of D-enzyme from amylomaltase on Bio-Gel P-150. Transferases from Step 4 (2.5 ml) were applied to the column (110 × 2.5 cm) in 70 mM sodium phosphate (pH 7.0). The column was eluted with the same buffer (flow rate 8 ml per h), and 3.0-ml fractions were collected. The fractions of the hatched peaks of activity were separately pooled to give peak A (D-enzyme) and peak B (amylomaltase). ———, % transmittance at 280 nm; •———•, glycosyltransferase activity.

weights. Chromatography on hydroxyapatite increased the specific activity about 2—3.5-fold. Amylomaltase was eluted with 75 mM sodium phosphate, whereas 90 mM sodium phosphate buffer was required to elute the D-enzyme from the hydroxyapatite column. This final purification step yielded a specific activity of 391 mU/mg protein for the D-enzyme, corresponding to a 98-fold purification over the initial extract. Amylomaltase was purified 248-fold, and the specific activity of the pure enzyme was 990 mU/mg protein (Table I).

Rechromatography of the purified glycosyltransferases (3.0 mg) from Step 6 on a column (60 × 0.9 cm) of fine particle-sized hydroxyapatite revealed a single symmetrical peak of protein and activity for each enzyme, indicating that we had obtained homogeneous enzyme preparations. The purified enzymes were free of any maltodextrin phosphorylase and protease activity. As described later, the enzymes were also judged to be pure by polyacrylamide gel electrophoresis in the presence of SDS.

The activity of the purified glycosyltransferases was found to decline gradually when the preparations were held in dilute solutions at 0—4°C. However, no significant loss of activity was observed for at least one month when the enzyme preparations were stored as a suspension in 3.2 M ammonium sulfate at 4°C.

*Molecular weight*

The molecular weights of the pure amylomaltase and D-enzyme were estimated by gel chromatography on a calibrated column of Sephadex G-200. The

molecular weight of the amylomaltase was found to be 74 000. This value is in agreement with the molecular weight value of 71 000 reported for the amylomaltase of *E. coli* [17,18]. The molecular weight of the pure D-enzyme was estimated to be 115 000. Molecular weight values for D-enzymes from other sources have not been published.

Gel electrophoresis of the denatured enzymes in the presence of SDS showed two protein bands for each enzyme preparation. The two protein bands of the D-enzyme had molecular weights of 63 000 and 52 000, respectively. Assuming that the D-enzyme is a dimer composed of two different subunits, these values are a good support for the molecular weight of the native D-enzyme estimated by gel chromatography. SDS-gel electrophoresis of amylomaltase yielded two protein bands, corresponding to molecular weights of 47 000 and 38 000, respectively. Since no evidence was found by this technique for any contaminant in the purified enzyme preparations, we conclude that both enzymes were pure and are probably composed of two different subunits.

*Effect of temperature and pH on enzyme activity*

The amylomaltase of *Ps. stutzeri* showed a relatively broad temperature activity curve, and the maximal activity occurred at 37°C. The enzyme was rapidly inactivated at temperatures above 50°C. The D-enzyme was most active at 30°C. The activity decreased rapidly above 37°C and was almost completely lost at 42°C.

The optimum pH for amylomaltase from *Ps. stutzeri* was at pH 7.7 in 0.1 M phosphate buffers, with a substantial amount of activity at pH 9.0 (80% of maximal activity). A quite different pH profile has been reported for the *E. coli* amylomaltase [19], with the optimum at pH 6.9. In 0.1 M phosphate buffers the optimum pH for D-enzyme activity was 7.6. This value is much higher than those reported for plant D-enzymes [20,21].

*Action pattern of amylomaltase and D-enzyme*

The action pattern of amylomaltase from *E. coli* ML has been extensively studied by several workers [17—19,22,23]. It has been demonstrated that amylomaltase is a non-specific 4-α-glucanotransferase with broad glycosyltransfer specificity. The enzyme catalyzes the transfer of both glucosyl and maltodextrinyl residues from the nonreducing end of a maltodextrin donor to an acceptor molecule [18]. According to the proposed mechanism of action a random distribution of maltodextrins should be expected in the reaction mixture after extensive incubation.

Characteristic action patterns for amylomaltase of *E. coli* and *Ps. stutzeri* were obtained by gel chromatography of the maltodextrin reaction mixtures on Bio-Gel P-2. The action of amylomaltase from *E. coli* on maltotetraose is shown in Fig. 4A. Due to transfer of both glucosyl and 4-α-glucanosyl units of varying chain-length a series of homologous 1,4-α-glucans were formed. In the equilibrium state of this reaction, successive transglycosylation led to the formation of 1,4-α-glucans with a degree of polymerization greater than 25 (higher dextrins peak). Incubation of amylomaltase from *Ps. stutzeri* with maltotetraose yielded a similar pattern of maltodextrins (Fig. 4B) as formed by the action of amylomaltase from *E. coli*. Unlike the *E. coli* enzyme, the amylomaltase of

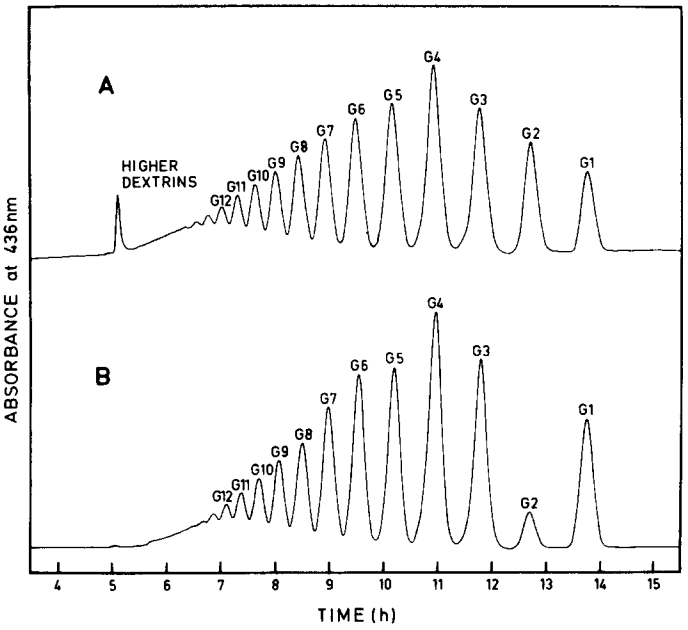


Fig. 4. Gel chromatographic separation of glucose and maltodextrins, synthesized by the action of amylomaltase from *E. coli* ML 30 (A) and *Ps. stutzeri* (B) on maltotetraose. Maltotetraose (20 mg per ml) was incubated with the purified enzymes as described in Materials and Methods. Chromatography of carbohydrates was carried out on a column (200 × 1.5 cm) of Bio-Gel P-2, minus 400 mesh at 65°C. The column was eluted with degassed water at a flow rate of 24 ml per h. 90-µl aliquots of 2% solutions were applied to the column. Peaks G1, G2, G3, etc. denote glucose, maltose, maltotriose, etc. Carbohydrates in column effluent were detected by the orcinol-sulfuric acid method using an autoanalyzer.

*Ps. stutzeri* formed only traces of higher dextrins (degree of polymerization greater than 25), and the amount of maltose in the reaction mixture was apparently lower (Fig. 4B). The chromatograms in Fig. 5 illustrate that comparatively small amounts of maltose were also formed by the action of *Ps. stutzeri* amylomaltase on maltotriose (Fig. 5A) and maltopentaose (Fig. 5B). The relatively small release of maltose suggests that amylomaltase of *Ps. stutzeri* does not readily act on the linkage penultimate to the reducing end of the maltodextrin donor molecule.

The chromatogram in Fig. 5A shows a remarkable high maltopentaose peak deriving from the action of amylomaltase on maltotriose. It may be concluded that the initial step in this reaction is the transfer of a maltosyl residue from a maltotriose donor to a maltotriose acceptor molecule. The remaining high maltotriose peak which appeared even on prolonged incubation (Fig. 5A) indicates that the enzyme acts on maltotriose at a relatively low rate. As shown later in this paper, this was also confirmed by kinetic studies.

Chain-lengthening transfer occurred with maltotriose, maltotetraose, and maltopentaose as initial substrates. However, no essential chain-lengthening reaction could be observed with maltohexaose as the initial substrate (Fig. 5C). It was rather surprising that the *Ps. stutzeri* amylomaltase acted on maltohexaose to form all products from glucose to maltopentaose, but no significant

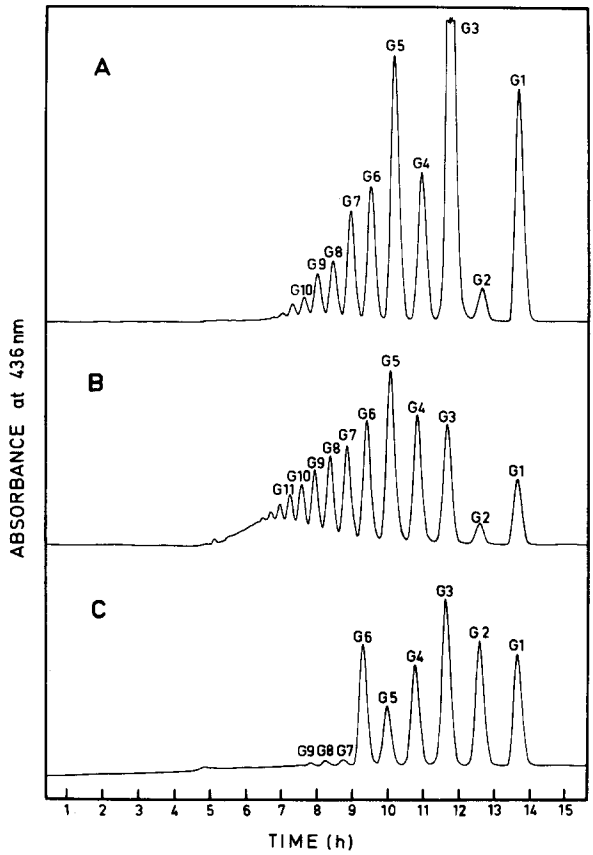


Fig. 5. Action of amylomaltase from *Ps. stutzeri* on maltotriose (A), maltopentaose (B), and maltohexaose (C). Glucose and maltodextrins were separated on a Bio-Gel P-2 column. For other details see the legend to Fig. 4.

amount of polymers larger than the initial maltodextrin substrate were formed. Obviously, maltohexaose is a good donor substrate, but this oligosaccharide seems to be unable to function as an acceptor. Thus, an elution profile was obtained with this substrate, which resembles a typical degradation pattern. Extensive incubation of amylomaltase with purified maltose and subsequent gel chromatography yielded besides a high maltose peak only traces of glucose but no detectable amounts of higher oligosaccharides.

Recent studies on the disproportionating enzyme of potato [20,24] revealed that this glycosyltransferase acts more specific on its substrates than does amylomaltase. The potato D-enzyme splits a maltodextrin donor substrate and transfers that portion of the oligosaccharide which contains the nonreducing end to an acceptor molecule. The reducing end of the maltodextrin donor is released predominantly as glucose. Thus, the initial substrates are disproportionated into products of higher and lower molecular weight [3].

Disproportionation of maltotriose with *Ps. stutzeri* D-enzyme is shown in Fig. 6A. Maltotriose was acted on to give the following major products of reac-

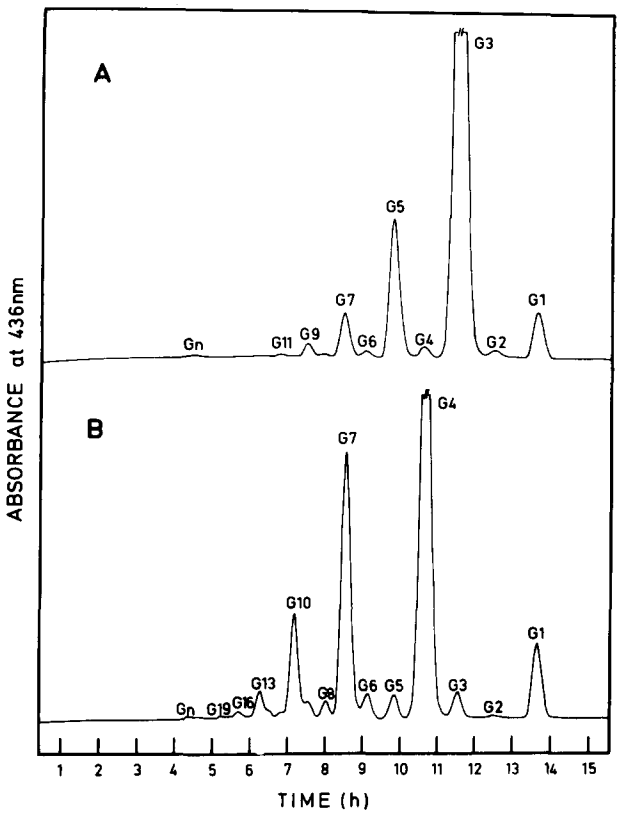


Fig. 6. Action of D-enzyme from *Ps. stutzeri* on maltotriose (A) and maltotetraose (B), and gel chromatography of the reaction products on Bio-Gel P-2. For other details see Fig. 4.

tion, according to the proposed scheme:

maltotriose + maltotriose ⇌ maltopentaose + glucose (1)

maltopentaose + maltotriose ⇌ maltoheptaose + glucose (2)

maltoheptaose + maltotriose ⇌ maltononaose + glucose (3)

maltononaose + maltotriose ⇌ maltoundecaose + glucose (4)

In the above equations maltotriose, as the initial substrate, is the acceptor molecule. Analogous to the above reaction scheme, D-enzyme of *Ps. stutzeri* formed from maltotetraose as the initial substrate glucose, maltoheptaose, maltodecaose, etc., as the major products (Fig. 6B). The occurrence of some minor products of reaction may be explained by non-specific cleavage of the maltodextrin donor molecule under our conditions of extensive incubation. As can be further deduced from Fig. 6, the rate of transfer decreases with increasing chain-length of the glucanosyl unit transferred. The maximum size of the glucanosyl chain that can be transferred by D-enzyme from *Ps. stutzeri* has not yet been established.

With maltopentaose and maltohexaose as the initial substrates, the D-enzyme

formed appreciable amounts of maltononaose and maltoundecaose by transferring maltotetraosyl and maltopentaosyl residues, respectively. The reaction of D-enzyme with highly purified maltose yielded only glucose and small amounts of maltotriose which indicates that the transfer of glucosyl units by

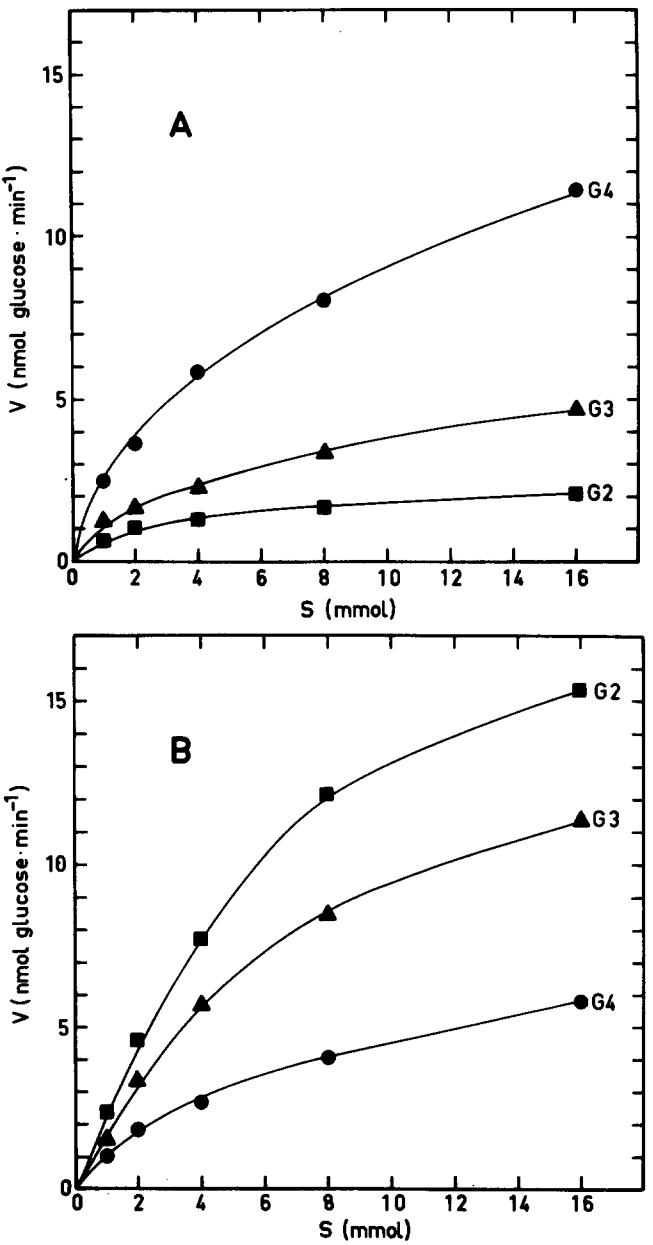


Fig. 7. Plot of the reaction velocity, $V$, as a function of the substrate concentration, $S$, for amylomaltase (A) and D-enzyme (B) with maltose (■, G2), maltotriose (▲, G3), and maltotetraose (●, G4). The incubation mixtures (1.2 ml; 30° C) contained varying amounts of maltodextrins in 0.1 M phosphate (pH 7.6), 10.2 mU amylomaltase or 5.8 mU D-enzyme, respectively. Release of glucose was determined as described in Materials and Methods.

this enzyme occurs at a very low rate. As shown below, the release of glucose from maltose was catalyzed by D-enzyme at a higher rate than from other maltodextrins examined. In this respect the bacterial enzyme differs from plant D-enzyme. Maltose is not a donor substrate for potato D-enzyme, and this sugar has relatively weak acceptor activity [20].

None of the products synthesized by *Ps. stutzeri* amylomaltase and D-enzyme were of sufficient length to stain blue with iodine. Evidence for the exclusive formation of 1,4-α-glucosidic bonds was obtained by treatment of maltotetraose with D-enzyme or amylomaltase and subsequent degradation of the reaction products with β-amylase. Gel chromatography on Bio-Gel P-2 and gas-liquid chromatography of the hydrolyzates yielded maltose as the main product. Glucose and maltotriose were formed in minor amounts. The presence of a homologous series of maltodextrins was further established by plotting the negative logarithm of the distribution coefficient of the oligosaccharides against their molecular weight [8]. A straight line was obtained for the reaction products of both *Pseudomonas* glycosyltransferases, as was the case with a homologous series of maltodextrins formed by *E. coli* amylomaltase [25].

*Kinetic properties*

The action of amylomaltase and D-enzyme from *Ps. stutzeri* on varying amounts of maltodextrins is shown in Fig. 7. Of the maltodextrins tested, amylomaltase was most active with maltotetraose as substrate, whereas maltotriose turned out to be a poorer substrate for this enzyme (Fig. 7A). Double reciprocal Lineweaver-Burk plot yielded a $K_m$ for maltotetraose of 7.1 mM. Chromatographically pure maltose was a very poor substrate for the *Ps. stutzeri* amylomaltase (Fig. 7A). The restricted role of maltose as a substrate for amylomaltase has been also reported for the corresponding enzyme of *E. coli* [18], 22]. On the other hand, the glucose release from maltose was catalyzed by the D-enzyme at a much higher rate than from maltotriose and from maltotetraose (Fig. 7B). The $K_m$ value of D-enzyme with maltose as substrate was estimated to be 8.3 mM from a Lineweaver-Burk plot.

Amylomaltase was strongly inhibited by 1 mM $Hg^{2+}$ and $Zn^{2+}$ (approx. 80% inhibition), and also by $Fe^{2+}$ (50% inhibition). $Mg^{2+}$ and $Ca^{2+}$ did not stimulate or inhibit the enzyme reaction. The following compounds showed less than 20% inhibition: EDTA, *p*-chloromercuribenzoate (both at 1 mM), iodoacetamide (at 20 mM), and dithiothreitol (at 2.5 mM). Cyclomaltohexaose inhibited 25% at 10 mM, while cyclomaltoheptaose did not affect amylomaltase activity. While methyl-α-D-glucoside and phenyl-β-D-glucoside were reported to be competitive inhibitors of the *E. coli* amylomaltase [19], these carbohydrates (at 10 mM) were ineffective as inhibitors for the amylomaltase of *Ps. stutzeri.*

The D-enzyme was markedly inhibited by heavy metal ions. D-Enzyme activity was also inhibited by 50 mM Tris (35% inhibition) and 1 mM *p*-chloromercuribenzoate (30% inhibition). Dithiothreitol, 2-mercaptoethanol (both at 2.5 mM), and EDTA (at 1 mM) inhibited D-enzyme activity very slightly.

## Discussion

An inducible amylomaltase and D-enzyme have been purified approx. 100—250-fold from the crude cell extract of *Ps. stutzeri*. The purified enzymes were

homogeneous as evaluated by rechromatography on DNA grade hydroxyapatite and SDS-gel electrophoresis. Neither phosphorolytic nor proteolytic activities were detected in the purified enzyme preparations. The occurrence of amylomaltase and D-enzyme in one organism has not been described before.

Action patterns and some other properties revealed that the amylomaltase of *Ps. stutzeri* differs from the corresponding *E. coli* enzyme in optimum pH and temperature, and in the release of maltose from maltodextrin donor substrates. The *Pseudomonas* disproportionating enzyme resembles plant D-enzyme in several properties, although it can be readily distinguished from the latter by the utilization of maltose as donor substrate. Enzymes with a similar type of glycosyl-transfer activity have been also detected in *Bacillus subtilis* [26], in two *Streptococcus* species [27,28], and in animal tissues [29]. Of all the substrates tested, maltotetraose is the preferred substrate for amylomaltase of *Ps.*

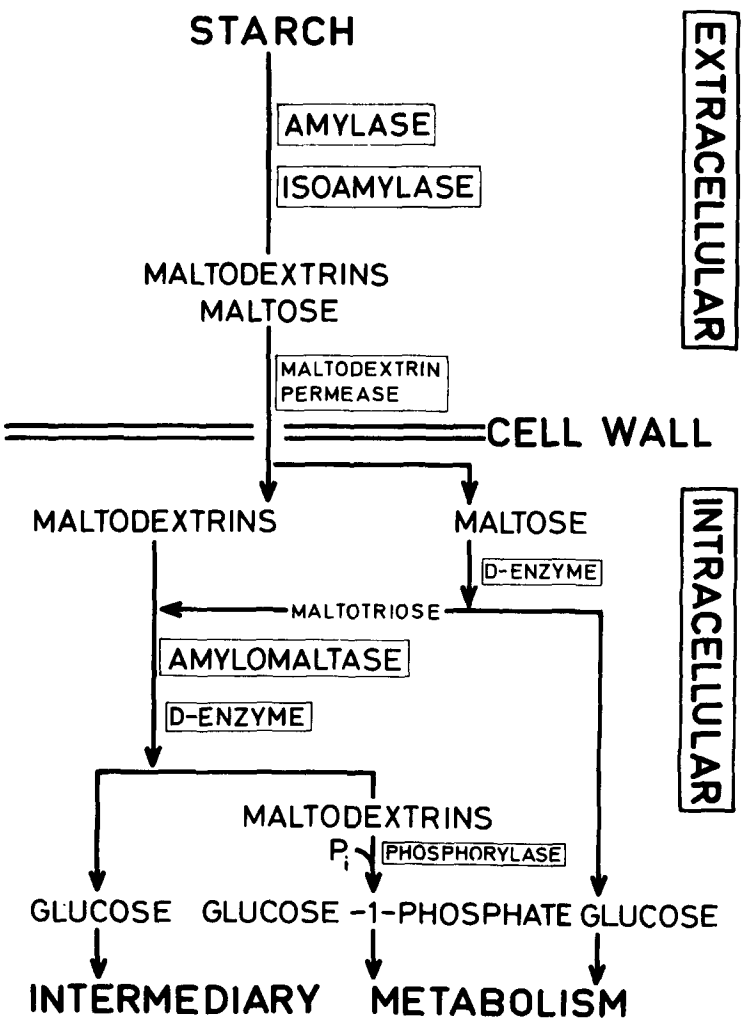


**Fig. 8. Proposed pathway for the utilization of starch by *Ps. stutzeri*.**

*stutzeri*. On the other hand, the glucose release by D-enzyme occurred at the highest rate with maltose as substrate.

Substrate specificity studies indicated the functional role of these enzymes in the starch-utilizing system of *Ps. stutzeri*. Several pathways for the metabolism of starch and glycogen in other bacteria have been proposed previously [30—32]. As completion of a pathway for the maltodextrin metabolism in *Ps. stutzeri*, which was proposed recently by Wöber [1], we suggest the scheme shown in Fig. 8. The enzymes involved in the extracellular metabolism of starch are the maltotetraose-forming amylase (1,4-α-D-glucan maltotetraohydrolase, EC 3.2.1.60), first described by Robyt and Ackerman [15], an isoamylase (glycogen 6-glucanohydrolase, EC 3.2.1.68), and maltodextrin permease [1]. Extracellular isoamylase activity was detected by the ability of the enzyme to debranch glycogen (Schmidt, J. and John, M., unpublished data). In contrast to previous findings [1], no pullulanase activity could be found in the culture medium of *Ps. stutzeri*. Maltodextrins arising from the action of extracellular amylases on starch are converted intracellularly into glucose and glucose 1-phosphate by the combined actions of amylomaltase, D-enzyme, and maltodextrin phosphorylase. Since maltose is a very poor substrate for amylomaltase, this sugar is converted by the action of D-enzyme into glucose and small amounts of maltotriose.

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