Purification and Partial Characterization of an Amylase from *Lysobacter brunescens*

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Four species of the genus *Lysobacter*, especially *L. brunescens* and *L. gummosus*, produced amylase when grown in liquid medium containing starch. The amylase was purified nearly 1200-fold with a 36% yield from the culture supernatant of *L. brunescens* by ammonium sulphate precipitation, CM-52 cellulose chromatography and gel filtration. An M_r of 47 000–49 000 was estimated for the enzyme from results of SDS-PAGE and glycerol gradient centrifugation, respectively. Metal ions such as Mg^{2+} , Ca^{2+} , Mn^{2+} or Zn^{2+} are not required for activity. The amylase is active from pH 5.0 to 7.5, degrades starch endohydrolytically and has a K_m of 2.08 mg starch ml^{-1} . Amylopectin, amylose and glycogen were also hydrolysed. Dextran, maltose, maltotriose, maltotetraose and 4-nitrophenyl α -D-glucoside were hydrolysed very slowly, or not at all. Other enzymes capable of degrading starch, maltose or malto-oligosaccharides were not detected in the culture supernatant of *L. brunescens*.

INTRODUCTION

The genus *Lysobacter*, order *Cytophagales* (Reichenbach, 1981) contains four recognized species, *L. antibioticus*, *L. brunescens*, *L. enzymogenes* and *L. gummosus*, as well as a number of strains or subspecies (Christensen & Cook, 1978). These organisms produce several extracellular enzymes (Jackson & Matsueda, 1970; Whitaker, 1970; Christensen & Cook, 1978; von Tigerstrom, 1980, 1981, 1983, 1984) as well as the antibiotic myxin and gummy polysaccharides (Christensen & Cook, 1978). All species we have investigated so far produce proteinases and peptides appear to be their preferred carbon and energy source. Carbohydrates such as monosaccharides are used slowly, particularly in the case of *L. enzymogenes* (unpublished). However, a number of polysaccharides are degraded by these organisms and *L. brunescens* appears to be particularly active in this regard (Christensen & Cook, 1978). Since most of the hydrolytic activities were determined by plate assays (Christensen & Cook, 1978), none of these polysaccharide-degrading enzymes have been investigated in detail. We selected *L. brunescens* for the study of an amylase. The production of this enzyme, its purification and some of its characteristics are reported in this paper.

METHODS

Chemicals. Soluble starch (S-516) was a product of Fisher. All other substrates, phosphorylase *b* and the glucose oxidase-peroxidase kit for the determination of glucose were purchased from Sigma. The protein standards were from Pierce. Catalase from bovine liver was obtained from Calbiochem and *Escherichia coli* C₄F₁ phosphatase was prepared in this laboratory (von Tigerstrom, 1983). Carboxymethylcellulose CM-52 (CM-52 cellulose) and Sephadex G-75 superfine were the products of Whatman and Pharmacia, respectively, and the TLC plastic sheets with silica gel 60 were from Merck.

Growth of the organisms. The *Lysobacter* species were obtained from Dr F. D. Cook, University of Alberta. Freeze-dried cultures were prepared for long-term maintenance. Short-term maintenance at 4 °C was done after growth at 20–22 °C on 0.4% tryptone, 1% (w/v) skim milk, 1.75% (w/v) agar. For the production of amylase, *L. brunescens* ATCC 29486 was grown for 48 h in 0.8% tryptone, 0.2% yeast extract, 0.3% starch, 2 mM-MgCl₂ at 20–

22 °C with shaking at 200 r.p.m. The initial and final OD₆₀₀ values were 0.03 and 5.3, respectively. The cells were removed by centrifugation at 16 300 g for 15 min and the culture supernatants were retained. The other organisms were treated in the same way.

Enzyme assays. Amylase activity was routinely determined at 37 °C by incubating 250 µl 1% (w/v) starch, 50 mM-Tris/HCl, pH 7.5, with up to 100 µl enzyme solution diluted with 50 mM-Tris/HCl, pH 7.5. The reactions were terminated by the addition of 500 µl dinitrosalicylic acid reagent (Miller, 1959). Colour was allowed to develop for 5 min at 95–97 °C and the A_{550} was determined after the addition of 2.5 ml H₂O. The A_{550} values were converted to µmol reducing sugar using glucose as the standard (A_{550} 2.2 = 1 µmol). Other polysaccharides were used as substrates where indicated. Rates of hydrolysis of maltose and other oligosaccharides were determined by detecting the amounts of glucose released. The hydrolysis of 4-nitrophenylglucosides was followed by monitoring the increase in A_{400} . Amylase activities are expressed in terms of units (U), where 1 U produces 1 µmol glucose equivalents min⁻¹. Catalase (Chance & Maehly, 1955), alkaline phosphatase (von Tigerstrom, 1984) and proteinase (von Tigerstrom, 1980) were assayed according to previously published procedures.

Glycerol gradient centrifugation. Step gradients consisting of 1.9 ml each of 30, 25, 20, 15, 10 and 5% (v/v) glycerol in 25 mM-Tris/HCl, pH 7.5, were allowed to equilibrate at 4 °C for 20 h. Samples of 0.3 ml containing 6 U amylase, 0.6 mg ovalbumin (M_r 45 000), 0.3 mg cytochrome *c* (M_r 12 500), 45 µg catalase (M_r 230 000) and 0.2 units *E. coli* alkaline phosphatase (M_r 84 000) were applied to the gradients. The preparations were centrifuged at 4 °C in a SW40 Ti rotor at 202 000 g_{av} for 24 h. Fractions (approximately 0.5 ml) were collected from the bottom of the tubes and analysed for enzyme activity, A_{280} and A_{530} . The experiment was done twice in duplicate with minor variations and the results were in good agreement.

SDS-PAGE. This was done as described earlier (Maizel, 1971; von Tigerstrom & Stelmashuk, 1985) using phosphorylase *b* (M_r 97 000), BSA (M_r 67 000), ovalbumin (M_r 45 000), chymotrypsinogen (M_r 25 000) and cytochrome *c* (M_r 12 500) as the standard proteins. Starch, 0.05%, was included in the gels for determining amylase activity. These gels were incubated with 50 mM-Tris/HCl, pH 7.5, for varying lengths of time and stained with 0.1% I₂, 0.2% KI solution.

Analysis of products of the amylase reaction. At 37 °C, 400 µl 1% starch in 50 mM-Tris/HCl, pH 7.5, were reacted with 375 µl amylase (17 U) in 50 mM-Tris/HCl, 0.5 mg BSA ml⁻¹, pH 7.5. At timed intervals, 100 µl samples of the reaction mixture were withdrawn, heated rapidly at 97 °C for 3 min to inactivate the amylase and cooled for storage. Samples (3 µl) were spotted on precoated silica gel sheets together with about 15 nmol each of standard glucose, maltose and malto-oligosaccharides. The silica gel sheets were developed using acetic acid/chloroform/acetone/H₂O (7:3:3:1, by vol.), a modification of the solvent used by Thirunavukkarasu & Priest (1984). The detection of the products has been described (Kelly *et al.*, 1986).

Analytical methods. Protein concentrations were determined by the Lowry method and by measuring the A_{280} (Warburg & Christian, 1941). Cytochrome *c* was detected by A_{280} and A_{530} measurements. The assay for NH₄⁺ has been described (von Tigerstrom, 1980).

Purification. All procedures were done at 0–4 °C. The method described below is representative of three similar purifications done at the same scale with slight modifications.

1. **Ammonium sulphate precipitation.** Ammonium sulphate (1683 g) was dissolved in 3 l culture supernatant to obtain 80% saturation. The pH was adjusted to 5.0 with glacial acetic acid and the mixture was stirred slowly for 60 min. The precipitate was obtained by centrifugation at 25 900 g for 30 min and dissolved in 50 mM-Tris/HCl, pH 7.3, to a volume of 108 ml. This was dialysed against 3 × 2 l 50 mM-Tris/HCl, pH 7.3, and 137 ml were recovered.

2. **CM-52 cellulose chromatography.** The dialysed ammonium sulphate preparation was applied to a CM-52 cellulose column (4 × 22 cm) equilibrated with starting buffer (10 mM-Tris/HCl, 25 mM-KCl, pH 7.3). The column was eluted with 350 ml starting buffer and then with a linear gradient consisting of 600 ml starting buffer and 600 ml 10 mM-Tris/HCl, 0.6 M-KCl, pH 7.3. Fractions of about 18 ml were collected and assayed for amylase activity, A_{280} and salt concentration. The amylase in fractions 43–50 (151 ml) was pooled and concentrated to 5 ml by ultrafiltration using a Diaflo PM10 membrane.

3. **Gel filtration.** The concentrated amylase preparation was applied to a Sephadex G-75 superfine column (2.5 × 20.4 cm), equilibrated and eluted with 25 mM-Tris/HCl, 100 mM-KCl, pH 7.5. Fractions of 2.6 ml were collected and assayed for amylase activity and A_{280} . Fractions 35–39 were pooled and stored at –20 °C. The Sephadex column was calibrated with blue dextran, ovalbumin, cytochrome *c* and ammonium sulphate.

The purification is summarized in Table 1 and the chromatographic steps are shown in Fig. 1.

RESULTS AND DISCUSSION

All four species of the genus *Lysobacter* were grown initially as described in Methods to determine the amounts of amylase produced. *L. enzymogenes* ATCC 29487 and *L. antibioticus* ATCC 29479 had the lowest activities, 0.013 U per OD₆₀₀ unit, whereas *L. gummosus*

Fig. 1. Purification of *L. brunescens* amylase by CM-52 cellulose chromatography (a) and Sephadex G-75 gel filtration (b) as described in Methods (steps 2 and 3). Amylase activities (●), A_{280} (○) and KCl concentrations (---) were determined. Numbers 1–4 in part b indicate the points of elution of blue dextran, ovalbumin, cytochrome *c* and NH_4^+ , respectively.

Table 1. Purification of amylase from *L. brunescens*

The enzyme was purified as described in Methods. Corrections have been made for the removal of samples for activity and protein determination.

Purification step	Total protein (mg)	Total amylase (U)	Specific activity [U (mg protein) ⁻¹]	Purification (fold)	Proteinase activity ($\Delta A_{370} \text{ min}^{-1}$)
Culture supernatant	3990	1246	0.31	—	7200
(NH_4) ₂ SO ₄ precipitation	568	969	1.7	5.5	6588
CM-52 cellulose	63	987	15.7	51	930
Sephadex G-75 superfine	1.2	445	371	1197	≤ 1.2

ATCC 29489 and *L. brunescens* ATCC 29486 produced 0.073 and 0.066 U per OD₆₀₀ unit, respectively. Although *L. gummosus* had the highest activity, it was not chosen for further studies since it produces a very viscous culture supernatant which complicates the purification of extracellular enzymes. Further studies were carried out with *L. brunescens*. It grew with a generation time of about 4 h and produced the amylase after the exponential growth phase.

Amylase purification. *L. brunescens* produces several proteinases and other extracellular enzymes in addition to the amylase. A primary concern during the purification was the removal of the proteolytic activity from the amylase. The purification is summarized in Table 1 and the proteinase activities are also given for each step. Ammonium sulphate precipitation was used primarily as a means of concentrating the enzyme. Other methods such as ultrafiltration and ion exchange adsorption were not practical at this stage. This step also resulted in a five- to six-fold purification although most of the proteinase activity was retained. During dialysis, a precipitate, probably polysaccharide, was formed at low ionic strength. We found that about 50% of the enzyme was associated with this particulate material. The dialysed preparation was applied to the CM-52 cellulose column as a fine suspension, i.e. without centrifugation, to avoid loss of the adsorbed amylase activity. The ion exchange chromatography on CM-52 cellulose (Fig. 1a) resulted in a ninefold purification of the amylase and in a significant reduction of the proteinase

Fig. 2. SDS-PAGE of *L. brunescens* amylase. Lane 1 contains M_r standards (phosphorylase *b*, BSA, ovalbumin, chymotrypsinogen and cytochrome *c*) and lane 2 contains 75 μ l (5.6 μ g) amylase from purification step 3, dialysed against 10 mM-Tris/HCl, pH 8.0.

activity. The amylase was recovered in good yield. The fact that the amylase activity appears slightly lower in the previous step is due, in part, to inhibition of the enzyme by ammonium ions.

The last step, gel filtration on Sephadex G-75 superfine (Fig. 1*b*), resulted in an unusually good purification. Fortuitously, the amylase was retarded during the gel filtration and separated from other high- M_r substances. At this stage the amylase was nearly homogeneous and essentially free of protease activity. In the final preparation the amylase was purified 1237-fold and obtained in a 36% yield.

Retardation during Sephadex gel filtration has also been observed with an *Acinetobacter* amylase (Onishi & Hidaka, 1978). This might suggest that the Sephadex is a substrate analogue to which the enzyme binds reversibly. However, we found that the affinity of the amylase was even more pronounced with Sephacryl S-200. The amylase was eluted from Sephacryl S-200 very slowly, if at all, with buffer containing 100 mM-KCl; with 0.5 M-KCl, the enzyme was eluted

with poor recovery in a broad peak after two to three column volumes. Detergents and higher salt concentrations, such as 2 M-KCl, had no significant effect on the elution pattern. Attempts to use this adsorption phenomenon for a one-step purification have been unsuccessful so far.

M_r and purity. Since the results in Fig. 1(b) indicated an *M_r* much lower than that of cytochrome *c*, gel filtration could not be used to estimate the *M_r* of the amylase. Therefore, we used glycerol gradient centrifugation with catalase, *E. coli* alkaline phosphatase, ovalbumin and cytochrome *c* as the reference proteins. The amylase was eluted just before ovalbumin and an *M_r* of about 49 000 was estimated. The results of the SDS-PAGE (Fig. 2) were in agreement with this. The purified amylase had one major component with an *M_r* of about 47 000. Although we were unable to renature the protein to obtain amylase activity in polyacrylamide gels, we propose that the major protein band is due to the purified enzyme. The unusual behaviour of the enzyme on gel filtration and the absence of other protein bands support this assumption. Therefore, the amylase is likely to be composed of one polypeptide.

Treatment of the amylase with SDS at room temperature resulted in several activity bands upon PAGE. This probably was due to variable binding of the SDS to the enzyme, resulting in conformational isomers.

Optimum activity and stability of the amylase. The enzyme had a broad pH optimum with nearly maximum activity from pH 5.0 to 7.5. However, it was less stable at lower pH values and, therefore, the enzyme was assayed and stored at pH 7.5.

Incubation of the enzyme in 50 mM-Tris/HCl, 2 mM-EDTA, pH 7.5, at 20 °C for 10 min reduced its activity by 20–23%. An assay of this EDTA-treated amylase in a solution with excess 2 mM-MgCl₂, CaCl₂ or 1 mM-MnCl₂ or ZnCl₂ resulted in some reactivation of the enzyme. This was most pronounced with CaCl₂ where nearly all of the lost activity was regained. Addition of CaCl₂ to amylase not treated with EDTA resulted in little or no increase in activity.

We have not detected any unusual instability of the enzyme. For instance, at 75 µg ml⁻¹ in 25 mM-Tris/HCl, 100 mM-KCl, pH 7.5, or in 50 mM-Tris/HCl, pH 7.5, the enzyme was stable for at least 9 d at 0 or -20 °C. The diluted amylase (3.5 µg ml⁻¹) was much less stable under these conditions but inactivation of the enzyme could be prevented by the addition of 100 µg BSA ml⁻¹.

Incubation at 55 °C for 20 min inactivated 67% of the enzyme. This loss was reduced to 20% in the presence of 2 mM-CaCl₂ or 500 µg BSA ml⁻¹. The addition of both CaCl₂ and BSA prevented inactivation at 55 °C.

Substrate specificity, K_m and mode of action. Amylase activity was routinely determined with solubilized starch. With amylopectin the activity was 95% and with amylose or glycogen the activities were 65% of that obtained with starch. Dextran, maltose, 4-nitrophenyl α- or β-D-glucosides were not hydrolysed. The *K_m* for starch was determined with varying starch concentrations from 7.4 to 0.71 mg ml⁻¹ and found to be 2.08 mg ml⁻¹ after plotting the inverse of the reaction rates against the inverse of the substrate concentrations.

It was estimated that a purified amylase preparation with 36 U amylase ml⁻¹ produced only 80, 0.8, 2.3 and 29 nmol glucose equivalents min⁻¹ (ml enzyme)⁻¹ from starch, maltose, maltotriose and maltotetraose, respectively. Thus, the enzyme cleaves the α-1,4-glycosidic bonds of larger oligo- and polysaccharides. This mode of action was confirmed by the experiment shown in Fig. 3. Starch was hydrolysed with purified amylase and very little glucose was produced throughout the degradation. After 5 min four major products were present, maltose, maltotriose, maltotetraose and maltopentaose. Whereas the two larger oligosaccharides were degraded during continued incubation, maltose and maltotriose were degraded very slowly, if at all. Thus, these two products and presumably limit dextrans (not analysed) appear to be the end products of the amylase reaction. Similar results were obtained with crude amylase preparations indicating that the organism is incapable of degrading starch completely. Also, α-glucosidase activity in the culture supernatant was very low (1 × 10⁻⁴ U ml⁻¹). Thus, maltose and maltotriose probably are hydrolysed only intracellularly. Therefore, the amylase of *L. brunescens* is a typical α-amylase (Fogarty, 1983; Priest, 1984).

L. brunescens produces about as much amylase activity per cell as an amylase positive *Bacillus subtilis* (unpublished). However, the *Lysobacter* amylase probably will not become an enzyme of

Fig. 3. Analysis of starch degradation by *L. brunescens* amylase as described in Methods. Positions 1–6 contained glucose, maltose, maltotriose, maltotetraose, maltopentaose and maltohexaose, respectively. Position 7 was a control without starch and positions 8–14 were samples removed after 0, 1, 5, 10, 25, 180 and 630 min degradation, respectively.

commercial importance. Compared to established producers of amylase such as fungal and *Bacillus* species, the yield of about 1 mg l^{-1} is low and the enzyme lacks the desired heat stability and other properties important in industry. Thus, the enzyme described here is primarily of interest in the study of the physiology of *Lysobacter* species and related bacteria. Secondly, it is one of only a very few amylases reported from Gram-negative bacteria (Fogarty, 1983; Priest, 1984). Furthermore, the amylase is of interest since it is one of a number of extracellular enzymes that these Gram-negative bacteria excrete into the environment and the organism may be useful in elucidating the control of exoenzyme synthesis and the excretory process in Gram-negative bacteria.

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