

## Characterization of an Extracellular $\beta$ -Amylase from *Bacillus megaterium sensu stricto*

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*Bacillus megaterium sensu stricto* (NCIB 7581) produced amylase throughout exponential growth and during the early-stationary phase. Enzyme synthesis occurred in the absence of  $\alpha$ -glucans but the yield was maximal when malt extract or starch was supplied as carbon source. Of the nitrogen sources examined, soya flour stimulated the highest yield of amylase. The enzyme was susceptible to reagents that react with thiol groups and had an exo-action on starch yielding maltose with a  $\beta$ -anomeric configuration. It is concluded that the principal starch-hydrolysing enzyme from *B. megaterium* NCIB 7581 is a (1 $\rightarrow$ 4)- $\alpha$ -D-glucan maltohydrolase similar in its properties to other *Bacillus* and plant  $\beta$ -amylases.

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### INTRODUCTION

*Bacillus megaterium* is a diverse species consisting of at least two aggregates of strains (Gordon *et al.*, 1973). Those organisms that fail to accumulate nitrite from nitrate but deaminate phenylalanine represent *B. megaterium sensu stricto* while strains giving the opposite reactions conform to the description of *B. carotarum* (Gibson & Gordon, 1974). Although *B. carotarum* is often accepted as a synonym of *B. megaterium* (Smith *et al.*, 1952), recent numerical analyses (Logan & Berkeley, 1980; Priest *et al.*, 1980) and DNA homology studies (Claus & Hunger, 1980) clearly separate these taxa.

When grown on starch agar, *B. megaterium* produces a clear hydrolysis zone around the colony. *Bacillus carotarum* strains, on the other hand, are characterized by a weak zone of starch hydrolysis that retains some iodine-staining capacity (Gibson & Gordon, 1974), a feature that is consistent with  $\beta$ -amylase [(1 $\rightarrow$ 4)- $\alpha$ -D-glucan maltohydrolase] secretion. Furthermore, a soil bacterium hitherto identified as *B. megaterium* reduces nitrate to nitrite and in several other respects conforms to the description of *B. carotarum*. This organism secretes  $\beta$ -amylase (Higashihara & Okada, 1974). The starch-degrading enzymes from *B. megaterium sensu stricto* have not been characterized. Strain NCIB 7581 (ATCC 15374) has been studied by Gordon *et al.* (1973) and fits the description of *B. megaterium sensu stricto*. In this paper we show that strain NCIB 7581 secretes a  $\beta$ -amylase.

### METHODS

*Organism and growth conditions.* *Bacillus megaterium* NCIB 7581 was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen. *Bacillus megaterium* NRS 986 was provided by R. E. Gordon. Bacteria were maintained on nutrient agar (Oxoid) at 4 °C. Strain NCIB 7581 was grown in basal medium (Takasaki, 1976a) comprising (g l<sup>-1</sup>): K<sub>2</sub>HPO<sub>4</sub>, 3; MgSO<sub>4</sub>·7H<sub>2</sub>O, 1; biotin, 0.02 (pH 7.0); and supplemented as indicated in the text. Overnight cultures in nutrient broth (Oxoid no. 2) were used to inoculate indented flasks (500 ml) containing 50 ml medium and these were shaken (180 rev. min<sup>-1</sup>) at 30 °C on an orbital incubator for 22 h.

*Analytical procedures.* Protein was estimated by a modification (Miller, 1959) of the Lowry method.

Total carbohydrate was assayed by the phenol/sulphuric acid method (Dubois *et al.*, 1956). Paper chromatograms were developed in ethyl acetate/pyridine/water (10:4:3, by vol.) using Whatman no. 1 paper and the sugars were treated with silver nitrate reagent (Trevelyan *et al.*, 1951).

Amylase activity was estimated either by the increase in reducing power or by the decrease in iodine-staining of digests containing starch and culture supernatant solution. In the first method, cells were removed by centrifuging (10000 g for 10 min) and the supernatant solution was dialysed against several changes of phosphate buffer (0.01 M, pH 7.0) for 24 h at 4 °C. Dialysed crude extract was stable at 4 °C for at least 21 d. The assay mixture, comprising BDH potato starch [1 ml of a 1% (w/v) solution in 0.1 M-phosphate buffer, pH 7.0] and dialysed culture supernatant solution (1 ml) was incubated at 37 °C for 30 min. The reaction was halted by heating at 100 °C for 10 min before estimating the reducing sugars by a modification of the Nelson-Somogyi method (Robyt & Whelan, 1968). Controls were prepared using heat-inactivated enzyme and maltose was used as a standard. One unit of amylase activity was defined as the amount of enzyme which liberated 1  $\mu$ mol maltose  $\text{min}^{-1} \text{ml}^{-1}$  under the above conditions. For examination of the effect of the enzyme on the iodine-staining power of starch, assay mixtures were set up as above. Samples (1 ml) were removed at intervals and added to distilled water containing 1 M- $\text{H}_2\text{SO}_4$  (0.5 ml). Iodine solution (1 ml, containing 0.02 g  $\text{I}_2 \text{ l}^{-1}$  and 0.2 g  $\text{KI l}^{-1}$ ) was added, the volume was made to 25 ml with water and the absorbance was read at 600 nm against an appropriate water/iodine blank.

*Partial purification of enzyme.* The enzyme was partially purified from the supernatant of a culture incubated at 30 °C for 18 h. Culture fluid [40 ml, 860  $\mu$ g protein  $\text{ml}^{-1}$ , sp. act. 0.715 units (mg protein) $^{-1}$ ] was loaded on to a DEAE-cellulose (DE 52) column which had been equilibrated with 20 mM-citrate/phosphate buffer (pH 8.0) containing mercaptoethanol (10 mM). Fractionation was carried out using the above buffer and this resulted in elution of 7.3% of the total protein applied. Amylase activity was recovered as a major peak (fraction I) with a specific activity of 10.15 units (mg protein) $^{-1}$  representing a 14-fold purification. A minor peak (fraction II), which eluted shortly after fraction I, had a specific activity of 2.44 units (mg protein) $^{-1}$ . The total recovery of amylase activity was 68%.

*Enzyme characterization.* To measure the extent of degradation of starch and amylose, digests containing substrate (80 mg) in phosphate buffer (0.1 M, pH 7.0, 8 ml) and fraction I (8 ml, 272  $\mu$ g protein) or fraction II (8 ml, 240  $\mu$ g protein) were incubated at 37 °C for 48 h in the presence of toluene.

Maltosaccharides (5 mg) and maltosyl-Schardinger dextrin (10 mg) dissolved in water (100  $\mu$ l) were incubated with fraction I (10  $\mu$ l) or fraction II (10  $\mu$ l) for 24 h at 37 °C in an atmosphere of toluene. Products were analysed by paper chromatography.

Enzyme inhibition by *p*-chloromercuribenzoic acid (*p*-CMB) was studied according to Higashihara & Okada (1974). A mixture containing enzyme (1 ml), citrate buffer (0.1 M, pH 4.6, 0.6 ml) and *p*-CMB solution (1 mM, 0.4 ml) was incubated at 22 °C for 60 min. At intervals, samples (0.5 ml) were removed and assayed for amylase activity. Cysteine (10 mM, 1.25 ml) and citrate buffer (0.1 M, pH 7.0, 0.75 ml) were added to inactivated enzyme (0.5 ml). After incubation for 60 min at 22 °C the residual activity was estimated.

The mutarotation of the product of enzyme action was observed as the change in optical rotation with time. The reaction mixture contained fraction I (0.5 ml) and amylopectin (40 mg  $\text{ml}^{-1}$ ) in citrate buffer (10 mM, pH 6.0, 2.5 ml). After incubation at 22 °C for 17 h, the reaction was stopped by the addition of 0.1 ml  $\text{HgCl}_2$  (50 mg  $\text{ml}^{-1}$ ) and the mutarotation of the product was observed over 48 h.

*Chemicals.* Soya flour, yeast extract, corn steep liquor and waxy maize starch were kindly provided by ABM Chemicals, Stockport, Cheshire. All other medium ingredients were from Oxoid. Maltosaccharides were prepared according to Manners & Stark (1974) and maltosyl-Schardinger dextrin was obtained from amylopectin  $\beta$ -amylolysis limit dextrin by the action of *Bacillus macerans* amylase. Amylose and amylopectin were prepared from broad beans by R. W. Gordon. Laminarin (from *Laminaria hyperborea*), cellodextrin and barley  $\beta$ -glucan were laboratory samples obtained from D. J. Manners. Pullulan was prepared from *Aureobasidium pullulans*.

## RESULTS AND DISCUSSION

The production of amylase when *B. megaterium* was grown in batch culture in basal medium containing peptone (1%, w/v) and malt extract (0.6%, w/v) is shown in Fig. 1. Enzyme production accompanied exponential growth and continued into the early-stationary phase reaching a maximum yield 12 h after exponential growth had ceased. During growth, the pH of the culture dropped initially and subsequently increased to reach pH 7.8 after 24 h incubation. This pattern of amylase production was similar to that reported for *Bacillus cereus* var. *mycoides* (Shinke *et al.*, 1975a; Takasaki, 1976b) and *Bacillus polymyxa* (Griffin & Fogarty, 1973) and contrasts with the typical pattern of  $\alpha$ -amylase synthesis exemplified by *Bacillus subtilis* and *Bacillus amyloliquefaciens* in which

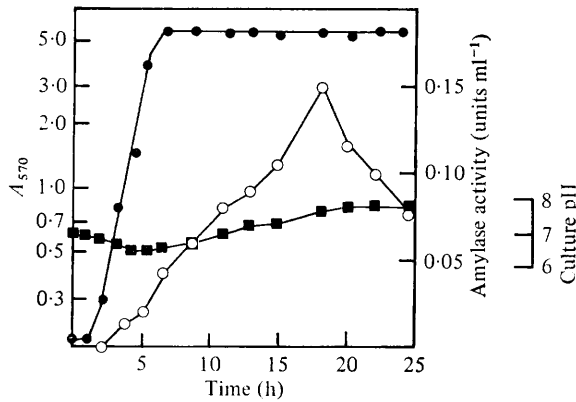


Fig. 1. Growth of *B. megaterium* and extracellular amylase secretion in basal medium containing peptone (1%, w/v) and malt extract (0.6%, w/v): ●, culture density; ○, amylase activity in medium; ■, pH of culture.

Table 1. Effect of nitrogen source on amylase synthesis by *B. megaterium*

The basal medium was supplemented with starch (0.5%, w/v) and cultures were incubated for 20 h.

Nitrogen source (2%, w/v)	Biomass (mg dry wt ml <sup>-1</sup> )	Amylase activity	
		10 <sup>3</sup> × units ml <sup>-1</sup>	10 <sup>3</sup> × units (mg biomass) <sup>-1</sup>
Soya flour	2.4	68.6	28.6
Peptone	2.4	33.8	14.1
Lab-lemco	2.4	30.9	12.9
Casein hydrolysate	2.0	18.2	9.1
Skim milk	1.8	16.4	9.1
Yeast extract	2.1	14.9	7.1
Corn-steep liquor	2.8	10.4	3.7
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	1.3	6.7	5.2
Urea	1.4	5.7	4.1

maximum enzyme secretion occurs during the stationary phase (Priest, 1977). The rapid decline in amylase activity which occurred during stationary phase was probably the result of proteolytic degradation.

Amylase was produced by strain NCIB 7581 irrespective of the nitrogen source (Table 1), although the yield was lower with less complex media. Soya flour stimulated production of amylase activity and a concentration of 1% (w/v) was found to be optimal. Similarly *B. cereus* var. *mycooides* (Takasaki, 1976a) and *B. polymyxa* (Griffin & Fogarty, 1973) require a complex nitrogen source for optimal  $\beta$ -amylase production. There are no equivalent data for  $\beta$ -amylase synthesis by *B. megaterium* no. 32 (Higashihara & Okada, 1974).

The enzyme was synthesized irrespective of the carbon source composition but the more complex materials provided greater yields (Table 2). Malt extract and starch stimulated maximum production and the optimum concentration of malt extract was found to be 0.6% (w/v).  $\beta$ -Amylase synthesis in the absence of exogenous  $\alpha$ -glucans is exceptional since in both *B. cereus* var. *mycooides* (Takasaki, 1976a) and *B. polymyxa* (Griffin & Fogarty, 1973)  $\beta$ -amylase is apparently inducible and its synthesis is dependent on the presence of starch or malto-oligosaccharide in the medium.

Table 2. *Effect of carbon source on amylase synthesis by B. megaterium*

The basal medium was supplemented with soya flour (1%, w/v) and cultures were incubated for 20 h.

Carbon source (0.5%, w/v)	Biomass (mg dry wt ml <sup>-1</sup> )	Amylase activity	
		10 <sup>3</sup> × units ml <sup>-1</sup>	10 <sup>3</sup> × units (mg biomass) <sup>-1</sup>
Malt extract	1.4	57.4	41.0
Maize starch	1.3	46.0	35.4
Potato starch	1.3	34.0	26.2
Maltose	1.3	33.1	25.5
Glucose	1.4	30.8	15.7
Sucrose	1.2	9.0	7.5
Lactose	1.5	7.5	5.0
Glycerol	1.5	3.0	2.0

#### *Properties of the enzyme*

Some properties of the crude enzyme (dialysed culture supernatant) were examined initially. The amylase activity was maximal at pH 6.5 to 7.0 and decreased to about 38% of the maximum at pH 5.5. The enzyme was stable between pH 5.0 and 9.0 and activity was lost completely after incubation at 30 °C for 3 h at pH 3.0 in 0.2 M-citrate buffer. This pH-activity profile is similar to those of other *Bacillus*  $\beta$ -amylases (Higashihara & Okada, 1974; Shinke *et al.*, 1975*a, b*; Fogarty & Griffin, 1975; Takasaki, 1976*b*), particularly the reduced activity and instability at low pH. Plant  $\beta$ -amylases, on the other hand, generally show maximum activity around pH 5.0 (French, 1960).

The optimum temperature for amylase activity was about 50 °C under the standard assay conditions and above this temperature activity declined sharply. This is similar to other bacterial  $\beta$ -amylases (Higashihara & Okada, 1974; Shinke *et al.*, 1975*b*; Takasaki, 1976*b*) with the exception of the *B. polymyxa* enzyme which showed maximal activity at 37 °C with only 20% activity remaining after 1 h at 45 °C (Fogarty & Griffin, 1975). These bacterial enzymes are therefore less thermostable than their plant counterparts (Robyt & Whelan, 1968).

The dialysed supernatant was tested for some other hydrolytic activities and found to be inactive against the following substrates: *p*-nitrophenyl  $\alpha$ -D-glucopyranoside, maltose,  $\alpha, \alpha$ -trehalose,  $\beta, \beta$ -trehalose, cellobiose, cellodextrin, laminarin,  $\beta$ -glucan and pullulan. It did not synthesize sugars from glucose but it did attack starch to yield maltose. The major carbohydrate-metabolizing activity was therefore  $\beta$ -amylase.

The enzyme was partially purified by ion-exchange chromatography; major (I) and minor (II) fractions were recovered. These activities were further characterized. The extent of degradation of starch and amylose by an enzyme is an important criterion of its action type (Manners, 1962). The extent of conversion of starch to maltose was 57% for fraction I and 60% for fraction II and this degradation was accompanied by a decrease in the iodine-staining power of 58% in each case. Amylose was degraded by 72% (fraction I) and 70% (fraction II), very similar to the 70% hydrolysis of potato amylose by crystalline plant  $\beta$ -amylase (Robyt & Whelan, 1968). Amylolysis of starch by exo-attacking enzymes is accompanied by a large release of reducing power but relatively little reduction of the iodine-staining capacity of the substrate. Conversely, endo-attacking enzymes rapidly reduce the iodine-staining capacity but release relatively little reducing power. The action pattern for fractions I and II (Fig. 2) followed the exo-pattern, typical of a  $\beta$ -amylase. A curve for the endo-attacking enzyme,  $\alpha$ -amylase from *Bacillus licheniformis* NCIB 6346, is included for comparison.

The action patterns of fractions I and II on malto-oligosaccharides were consistent with

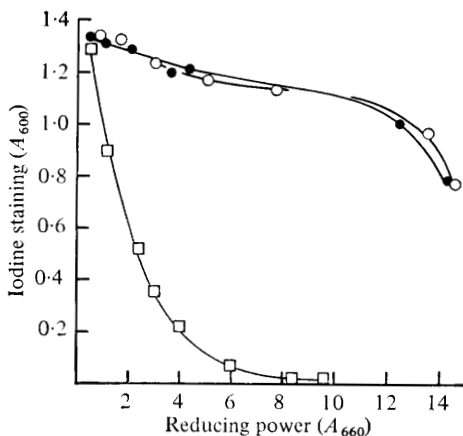


Fig. 2. Release of reducing power from starch and reduction of iodine-staining capacity during hydrolysis by  $\beta$ -amylase. Starch was incubated for 48 h with: ●, fraction I; ○, fraction II; □, culture supernatant from *B. licheniformis* NCIB 6346. For details, see text.

the splitting of maltosyl units from the non-reducing ends of the molecules. Thus, maltotetraose was hydrolysed slowly to maltose, and maltopentaose yielded maltose and maltotriose. Higher maltosaccharides yielded maltose as the predominant product. In a similar experiment, there was no action by either fraction on maltosyl-Schardinger dextrin indicating that debranching activity was absent.

Ion-exchange chromatography of dialysed culture supernatant in the absence of mercaptoethanol resulted in major loss of amylase activity suggesting that the thiol groups of the protein were required in reduced form for stability and activity. This was further analysed by assessing enzyme inhibition by *p*-CMB. Within 30 min at 22 °C, enzyme activity was reduced to 25% of its original value by treatment with 0.5 mM (final concn) *p*-CMB. After 60 min the activity was reduced to 15%. Cysteine reactivated the enzyme to 65% and 44%, respectively. In this respect, the  $\beta$ -amylase from strain NCIB 7581 resembled other bacterial (Higashihara & Okada, 1974; Fogarty & Griffin, 1975; Takasaki, 1976*b*) and plant (French, 1960)  $\beta$ -amylases which are similarly inactivated by *p*-CMB.

Final verification that strain NCIB 7581 was producing a  $\beta$ -amylase was obtained by measuring the mutarotation of the maltose produced from amylopectin over 48 h. The upward change in optical rotation indicated that the maltose was in fact initially produced with a  $\beta$ -configuration.

Since *B. megaterium* NCIB 7581 and *B. megaterium* no. 32, which conforms to the description of *B. carotarum* (Gibson & Gordon, 1974), both secrete  $\beta$ -amylase, some explanation is required to account for their different hydrolysis reactions when grown on starch nutrient agar. Evidence for secretion of the debranching enzyme pullulanase can be readily obtained by incubating cultures on pullulan agar (Morgan *et al.*, 1979). Strain NCIB 7581 and several other strains of *B. megaterium sensu stricto* readily hydrolysed the pullulan in this medium indicating that they synthesized a pullulanase. The absence of a pullulan-hydrolysing activity in the culture supernatant from strain NCIB 7581 (see above) suggests that either the cultural conditions were not suitable for pullulanase synthesis (or secretion) or that the enzyme was labile and denatured during the 24 h dialysis period. Strain NRS 986, on the other hand, which was recently classified as '*B. carotarum*' (Priest *et al.*, 1980), failed to hydrolyse the pullulan in pullulan agar after incubation for 4 d at 30 °C. It is possible, therefore, that the clear hydrolysis zone observed after growth of *B. megaterium sensu stricto* on starch agar may be due to the combined action of a  $\beta$ -amylase and pullulanase while the hazy zones from strains conforming to *B. carotarum* (Gibson &

Gordon, 1974) may be the result of  $\beta$ -amylolysis of the starch yielding  $\beta$ -limit dextrins. We are currently studying the extracellular enzymes from strains of *B. megaterium* and related taxa to characterize the various  $\alpha$ -glucan-hydrolysing systems more fully.

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