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The purification of a novel amylase from *Bacillus subtilis* and its inhibition by wheat proteins

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A new α-amylase (EC 3.2.1.1) from *Bacillus subtilis* was purified by affinity chromatography. The molecular weight of the purified enzyme, estimated from sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, was 93000, which is very different from the molecular weights of two well-characterized amylases from *B. subtilis*. Electrofocusing showed an isoelectric point of 5. Amylase shows a broad maximum of activity between pH 6 and 7; maximal inhibition of enzyme by wheat-protein α-amylase inhibitors is displayed at pH 7.

The inhibition of α-amylase (EC 3.2.1.1) from very different species (Kneen & Sandstedt, 1943; Silano *et al.*, 1975) by wheat-seed proteins may have nutritional relevance (Macrì *et al.*, 1977), may provide seed with insect-resistance during ripening and storage (Silano *et al.*, 1975) and has been studied for the therapy of obesity and diabetes (Puls & Keup, 1973). Moreover, the system constituted by amylases and wheat-protein inhibitors offers a unique opportunity for the study of specific and reversible protein–protein interaction (Buonocore *et al.*, 1976, 1980; Silano, 1978; Silano & Zahnley, 1978).

A model describing the inhibition has been proposed (Silano *et al.*, 1977) on the basis of the inhibition of amylases purified from *Tenebrio molitor* larva (Buonocore *et al.*, 1975), chicken pancreas (Buonocore *et al.*, 1977) and octopus digestive gland (Belisario *et al.*, 1981) and of structural investigations that showed that most inhibitors belong to one of two families, one of monomeric proteins of molecular weight 12000, and the other of proteins of molecular weight 24000 that dissociate into two very similar 12000-dalton subunits (De Ponte *et al.*, 1976).

The model indicates that amylases have two binding sites for the wheat inhibitors (one for each inhibitor protomer); the binding energy is provided mainly by electrostatic charges, with a contribution by the inhibitor carbohydrate moiety interacting with some part of the amylase active site.

In an attempt to purify specifically the *Bacillus subtilis* saccharifying amylase, by means of wheat-protein-inhibitor-conjugated Sepharose, we obtained an amlyase different from other well-characterized amylases produced by *B. subtilis*.

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Experimental

The *Bacillus subtilis* strain used is classified as PB 3224 in the collection of Professor Ciferri, Istituto di Genetica, Pavia, Italy. Agar and peptone were from Costantino (Fauria, Turin, Italy). Inhibitor-conjugated Sepharose 4B, prepared as described elsewhere (Buonocore *et al.*, 1975), was kindly given by Professor V. Buonocore (University of Naples, Naples, Italy).

The 24000-dalton protein inhibitors were prepared from wheat flour as described by De Ponte *et al.* (1976).

Soluble starch was purchased from Carlo Erba (Milan, Italy). Acrylamide, bisacrylamide and sodium dodecyl sulphate were supplied by Merck (Darmstadt, West Germany). Standard proteins for molecular-weight determination were obtained from LKB Produkter (Stockholm, Sweden).

B. subtilis was grown under stirring and forced ventilation in a 50-litre tank for about 42h at 30°C in the following medium: agar, 1.5g/l, peptone, 6g/l, glucose, 10g/l, and yeast extract, 3g/l (pH 7). At the end of the fermentation the pH was 6.7. Liquid cultures were then centrifuged in a Sharples centrifuge at 15000rev./min. The clear supernatant was freeze-dried, stored at −20°C and used within 1 week.

Amylase activity was assayed with an iodine-staining method (Robyt & Whelan, 1968) at 37°C

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and at pH 6, in a 1 ml reaction mixture constituted of 50 mM-sodium cacodylate buffer, 1 mM-$CaCl_2$ and 0.1% freshly solubilized starch. Starch hydrolysis was linear up to at least 50% decrease in absorbance at 620 nm. One amylase unit is the activity needed for 50% decrease in the absorbance at 620 nm in a 10 min reaction period. For studies of pH-dependence of enzyme activity, succinate buffers (pH 4–6.5) and Tris buffers (pH 7–8.9) of constant ionic strength (I 0.01) were used (Perrin, 1963). Protein content was determined with a modification of the Lowry method (Oyama & Eagle, 1956), with bovine serum albumin as standard.

For the purification of amylase a slight modification of the affinity-chromatography method described by Buonocore *et al.* (1975) was used. Briefly, a column of inhibitor-conjugated Sepharose (4 ml bed volume, 1.5 cm diameter) was equilibrated with 20 mM-sodium barbital buffer, pH 7.0, containing 0.1 mM-$CaCl_2$. Portions (12 g) of the freeze-dried preparation were dissolved freshly at 10% (w/v) concentration in the equilibration buffer, spun at 18000 rev./min. in a B-20 centrifuge (International Equipment Co.) and immediately used as crude extract for adsorption on the column at a flow rate of 5 ml/h. After all the effluent (usually about 74 g) had emerged, the column was extensively washed with equilibration buffer until no protein could be detected in the eluate. Amylase was then eluted from the column with 20 mM-sodium barbital buffer, pH 9.0, containing 0.1 mM-$CaCl_2$. Eluted enzyme fractions were pooled, desalted on a Bio-Gel P-6 column (10 cm × 0.9 cm) equilibrated with water and freeze-dried.

Electrofocusing was performed at 4°C in a sucrose gradient; 1 ml of sample containing 55 units of purified amylase was mixed with Ampholine solution (either for the 5–8 or for the 3–10 pH range) and loaded on a semi-preparative LKB electrofocusing column; 0.25 M-NaOH and 0.25 M-H_2SO_4 were used as cathode buffer and anode buffer respectively. After a 48 h focusing, the Ampholine sample solution was eluted and the fractions were assayed for pH and amylase activity.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970) in a 12.5%-polyacrylamide 1.5 mm-thick slab gel containing 0.2% sodium dodecyl sulphate, at a constant current of 20 mA. Approx. 20 μg of purified α-amylase sample, containing 1% sodium dodecyl sulphate, with or without 0.1% dithiothreitol, was heated for 3 min at 95°C before being loaded on the gel. A molecular-weight-standard protein mixture was run beside the sample.

Results and discussion

The culture medium was assayed for amylase activity during the growth of the bacteria. Amylase activity increased linearly between 17 and 40 h of fermentation, reaching about 6 amylase units/ml of medium.

Table 1 summarizes quantitative aspects of the purification of the amylase.

The purified enzyme, when electrophoresed on polyacrylamide slab gel in the presence of 0.2% sodium dodecyl sulphate, exhibited a subunit molecular weight of 93000. Treatment with 0.1% dithiothreitol did not change the minimum molecular weight.

By this method the purity of the preparation was also checked (Fig. 1), and a band of very low molecular weight (approx. 10000) was apparent, which may represent peptone polypeptides still contaminating the sample.

pH-dependence of amylase activity showed a broad maximum at pH 6–7, with a 5 min reaction period. Activity determinations at different pH values after 30 min preincubation are shown in Fig. 2. They did not differ significantly from the non-preincubated-enzyme activities, except that at pH 5, where 46% irreversible decrease of activity occurred. Reversibility experiments showed also that after preincubation at pH 4 enzyme activity was lost completely. Enzyme activity at pH 7.0 in the low-ionic-strength (I 0.01) Tris buffer was lowered by 18% in the presence of either 50 mM- or 250 mM-KCl.

Isoelectrofocusing of the purified enzyme showed one main peak of enzyme activity with pI 5.0 and a secondary peak with pI 5.6.

The amylase residual activity at different pH

Table 1. *Purification of α-amylase from B. subtilis*
Mean values ± S.E.M. for five different purifications are given. For details see the text.

Fraction	Volume (ml)	Amylase activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	740	26456 ± 4506	32062 ± 742	0.83 ± 0.16	100	1
Eluate	740	12091 ± 1702	29552 ± 48	0.41 ± 0.06	—	—
Adsorbed	—	14365 ± 3516	—	—	52 ± 5	—
Desorbed	25	4440 ± 857	21 ± 7.4	347 ± 133	19 ± 5	442 ± 181

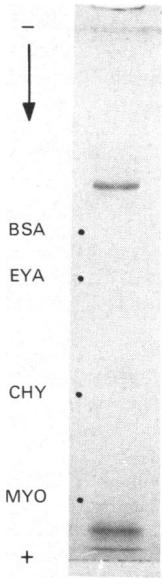

Fig. 1. *Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate of α-amylase purified by affinity chromatography from culture medium of B. subtilis PB 3224*

Experimental conditions are described in the text. Dots represent molecular-weight-standard proteins (BSA, bovine serum albumin; EYA, egg-yolk albumin; CHY, chymotrypsinogen; MYO, myoglobin).

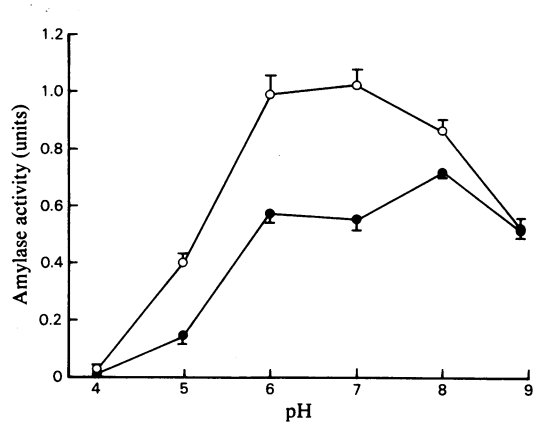

Fig. 2. *Activity of B. subtilis amylase at different pH values in the presence (●) or in the absence (○) of a wheat-protein inhibitor pool*

Enzyme was preincubated at the stated pH, with or without inhibitors, at 37°C for 30min before the addition of starch. Starch hydrolysis during a 10min reaction period was then assayed. Buffers of constant ionic strength (I 0.01) were used.

values after 30min preincubation with 0.4μg of protein of the 24000-dalton inhibitor family is shown in Fig. 2. Preincubation is needed because the inhibition reaction, shown to be reversible (Saunders & Lang, 1973; Buonocore *et al.*, 1975, 1976), is much slower than starch hydrolysis, and 10–30min preincubation is required for wheat inhibitor to show maximal activity with several amylases (Saunders & Lang, 1973; Buonocore *et al.*, 1976; O'Donnel & McGeeney, 1976). Inhibition takes place from pH8 down to acidic pH values. Because of denaturation effects, it was not possible to establish soundly the lower pH limit of inhibition on the pure amylase. pH-dependence of inhibition was therefore determined also on crude amylase, which is fairly stable in the assay conditions. In this case inhibition was apparent from pH8 to pH5. Inhibition of the pure enzyme at pH7 was not affected by KCl up to 250mM concentration.

The existence of different amylases from *Bacillus subtilis* strains has been known for a long time (Kneen & Beckford, 1946), and they have been differentiated into saccharifying and liquefying types according to their mode of catalytic action. Moreover, amylases of the former type can be inhibited by wheat-seed albumins.

A saccharifying amylase and a liquefying one have been extensively characterized (for a review see Takagi *et al.*, 1971); their subunit molecular weights are 41000 (Yutani *et al.*, 1969) and 47000–48900 (Fischer *et al.*, 1960; Kakiuchi, 1965) respectively. The amylase that we have purified therefore is clearly different from both in molecular weight.

Although we did not perform the appropriate tests as such (Kneen & Beckford, 1946; Fukumoto *et al.*, 1953; Okada *et al.*, 1968), the very low production of amylase by bacteria during fermentation and the inhibition by wheat-protein α-amylase inhibitors suggest that this new amylase belongs to the saccharifying type (Kneen & Beckford, 1946). Isoelectric point and pH–stability curves indicate that the enzyme is an acidic acid-unstable protein. A relevant inhibition by dimeric inhibitors takes place between pH5 and 7, i.e. between the isoelectric points of the amylase and of the major inhibitor of the pool used (De Ponte *et al.*, 1976); this protein, named 0.19-inhibitor from its mobility relative to Bromophenol Blue in polyacrylamide-gel electrophoresis at pH8.3, exhibits a pI of 7.3 (Sodini *et al.*, 1970). Although pH effects may reflect association–dissociation equilibria or conformational changes, the pH-dependence of the inhibition is consistent with the model of interaction proposed by Silano *et al.* (1977), which stresses the importance of attractive electrostatic forces in amylase inhibition.

In the present case, too, inhibition is significantly diminished or absent when both molecules carry the same net electric charge.

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