Activity and action pattern of *Bacillus licheniformis* α-amylase in aqueous ethanol

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A purified *B licheniformis* α-amylase in a mixture of ethanol-aqueous buffer (1 1, v/v) retains half the activity shown in water alone In ethanol-aqueous buffer (7 3, v/v) about 20% of the activity is retained The pattern of oligosaccharides produced from amylose changed with ethanol concentration, in aqueous buffer the products are DP 1 and 2, 33 7%, DP 3, 28 5%, DP 4, 4 4% and DP 5, 33.4% Whereas in ethanol-aqueous buffer (7 3, v/v) the products are DP 1 and 2, 66 8%, DP 3, 17 3%, DP 4, 4 1% and DP 5, 11 8% These results suggest that a change in substrate affinity at the active centre subsites is induced in the ethanol-aqueous buffer medium

Bacillus licheniformis *α-Amylase* *Enzyme activity* *Ethanol-aqueous buffer mixture* *Action pattern*

1. INTRODUCTION

The α-amylase (EC 3.2.1.1) from *Bacillus licheniformis* has been purified and characterised [1,2] and shown to have excellent thermostability. Its stability at temperatures above the gelatinization temperature of most starches has led to its wide use in industry for the initial hydrolysis of starch to dextrins which are then converted to glucose by glucoamylase [3]. For the same reason the enzyme is used in starch [4] and dietary fibre [5] analyses.

B. licheniformis α-amylase has been used to remove adventitious starch during plant cell wall purification [6]. In some cell wall isolation procedures, media such as 70% ethanol [7], glycerol, ethylene glycol-glycerol, 80% acetone and benzene-carbon tetrachloride (Harris, 1983) are used to prevent losses of water-soluble components. Although these media are not usually considered to be compatible with starch-degrading enzymes, α-amylase (porcine pancreas), β-amylase (sweet potato) and glucoamylase (*Aspergillus niger*) are all active in aqueous dimethyl sulphoxide [9] and this solvent has been used in a glucoamylase determination of starch [10]. We now report the activity of a purified *B. licheniformis* α-amylase in ethanol-aqueous buffer mixtures and the apparent changes in substrate binding and catalysis when ethanol concentration is increased.

2. MATERIALS AND METHODS

2.1. *Enzyme purification*

B. licheniformis α-amylase was purified [2] from Termamyl 60L a product of Novo Industri A/S, Bagsvaerd, Denmark.

2.2. *Isoelectric focussing*

Flat bed isoelectric focussing was performed in 7.5% acrylamide gels containing 2% carrier ampholytes. The carrier ampholytes were synthesised from triethylenetetramine, tetraethylenepentamine, pentaethylenehexamine (1:2:3, v/v) and acrylic acid using a nitrogen/carboxyl ratio of 2:1 [11]

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2.3. *Amylase assays*

A colorimetric assay based on the use of a dye-labelled starch (Phadbas, Pharmacia South Seas, Melbourne) was used to monitor fractions during enzyme purification and also to determine relative enzyme activity in aqueous ethanol. The pH of the assay medium was adjusted to 6.9 using succinate buffer [12]. An iodometric assay [13] using potato amylase (type II, Sigma, St.Louis, MO) was used to determine activity in aqueous buffers.

A reductometric assay was developed for use in aqueous ethanol buffers. Potato amylose (20 mg/ml, 4 ml) was mixed with buffer or buffer and ethanol (20 ml) and the pH maintained at 6.9 using glycerophosphate (20 mM) according to the pH* tables for partially aqueous solvents [14]. Enzyme solution (1 ml) previously adjusted to contain 3 ('dextrinizing power') enzyme units [13] was added to the buffered substrate samples incubated at 70°C with continuous agitation for 1 h. Enzyme digests were then heated at 100°C for 10 min, centrifuged (3000 × *g*), evaporated to a thin syrup under reduced pressure and diluted to 25 ml with water. Reducing sugars were determined on 100-μl samples using the *p*-hydroxybenzoic acid hydrazide (PAHBAH) reaction [15]. The PAHBAH reaction is not affected by ethanol [16].

2 4. *Viscometric enzyme assays*

(1→3)-β-Glucanase (EC 3.2.1.39), (1→4)-β-glucanase (EC 3.2.1.4) and (1→3,1→4)-β-glucanase (EC 3.2.1.73) were assayed using carboxymethyl-pachyman, carboxymethyl-cellulose and barley β-glucan substrates, respectively [17]

2.5. *Gel filtration*

Soluble oligosaccharides in enzymic digests of amylose were separated by gel filtration chromatography on Biogel P-2 (<400 mesh, BioRad Laboratories, Richmond, CA) [18]. The reaction mixtures were filtered (Whatman no.40), heated at 100°C for 10 min, centrifuged (3000 × *g*) and then evaporated at reduced pressure to thin syrups prior to injecting 5 μl onto the Biogel P-2 column. The column (9 mm × 1.7 m) was eluted with water at 2×10^5 N·m^{-2} pressure and a flow rate of 35 ml·h^{-1}. Column temperature was kept at 60°C. The column was calibrated using maltooligosaccharides (Boehringer Mannheim, North Ryde, NSW).

3. RESULTS

The crude Termamyl preparation hydrolysed carboxymethyl-pachyman and barley (1→3,1→4)-β-glucan but not carboxymethyl-cellulose. The purified preparations showed no activity on any of these substrates, gave a single band with a p*I* of 5.4 on isoelectric focussing and contained no carbohydrate as assessed by the alditol acetate procedure [19].

Fig.1 shows the effect of ethanol concentration on the activity of the purified α-amylase assayed using the colorimetric and reductometric procedures. The enzyme retained full activity measured reductometrically in (3:7, v/v) ethanol-aqueous buffer mixtures, more than 50% of its activity in (1:1, v/v) mixtures and 18% of its activity in (7.3, v/v) mixtures. The enzyme had no activity in ethanol-aqueous buffer (4:1, v/v). The colorimetric assay gave consistently lower relative activities than the reductometric assay.

The oligosaccharides produced by the enzyme from amylose on incubation in water for 24 h and in ethanol-aqueous (7:3, v/v) for 72 h are shown in figs 2 and 3, respectively. The extent of hydrolysis did not change after 8 h in water or after 60 h in ethanol-water (7:3, v/v). In water the DP 3 and 5 products predominate with smaller amounts of DP 1 and 2, as reported in [3], whereas in ethanol-water (7.3, v/v) DP 1 and 2 products

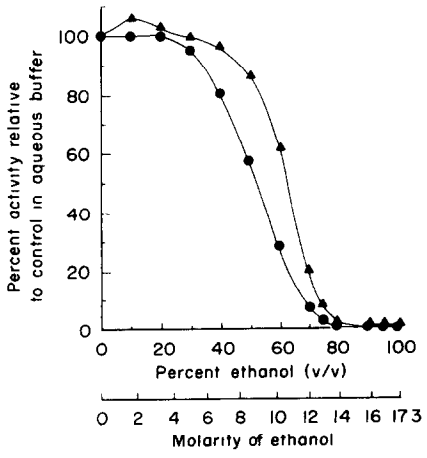

Fig.1 Relative activity of *B lichenformis* α-amylase in ethanol-water mixtures at pH 6.9* (●) Colorimetric assay, (▲) reductometric assay

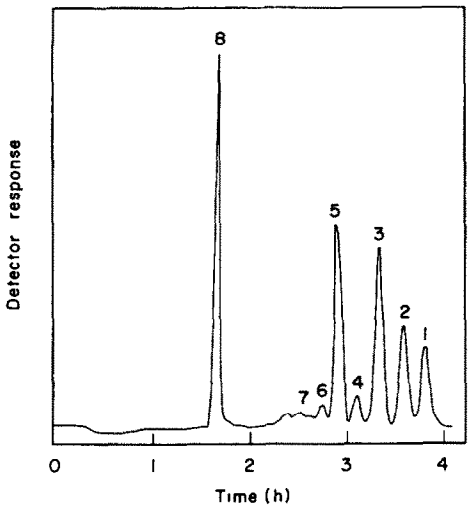

Fig 2. Bio-Gel P-2 elution profile of the oligosaccharides released from amylose by *B. licheniformis* α-amylase in aqueous buffer at pH 6.9: (1) glucose, (2) maltose; (3) maltotriose; (4) maltotetraose; (5) maltopentaose; (6) maltohexaose, (7) maltoheptaose, (8) salt

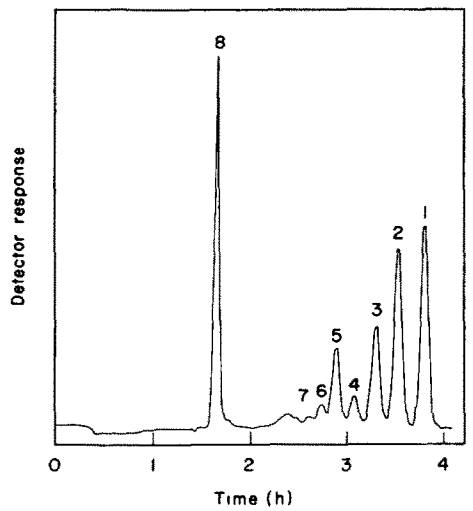

Fig.3. Bio-Gel P-2 elution profile of the oligosaccharides released from amylose by *B. licheniformis* α-amylase in ethanol-water (7·3, v/v) buffer at pH* 6.9: (1) glucose; (2) maltose; (3) maltotriose; (4) maltotetraose; (5) maltopentaose; (6) maltohexaose; (7) maltoheptaose; (8) salt.

are abundant, and there is less DP 3 and 5 product.

4. DISCUSSION

The activity of hydrolytic and other enzymes in organic solvent-water mixtures has been investigated sporadically, especially with reference to enzyme action at low temperatures and in immobilised states. Lysozyme is known to be active in a variety of mixed solvent systems [20] as is bovine pancreatic ribonuclease [21]. The present studies show that purified *B. licheniformis* α-amylase, which is known to be thermostable, is also active in ethanol-water mixtures. Whether the enzyme's thermal stability is related to this property is unknown. The decreasing activity of the enzyme as the ethanol concentration is raised can be attributed to a number of factors such as, the low water activity, lowered solubility of both enzyme and substrate, and denaturation of the enzyme by the organic solvent.

The higher relative activity recorded by the reductometric assay as compared with the colorimetric procedure (fig.1) may be related to the requirement of water to swell the cross-linked, dye-labelled starch substrate. As the ethanol concentration was increased the solid substrate showed a lower degree of swelling than in water thus decreasing its accessibility to the α-amylase

The difference in the nature of the ultimate hydrolysis products in ethanol-aqueous buffer (7 3, v/v) as compared with aqueous buffer is characterized by a relative increase in the DP 1, 2 and 3 products. This suggests that there has been a modification of the affinity at the binding sites so that in the ethanol-aqueous buffer mixture, binding and cleavage of oligomers to produce low DP products has been favoured. Subsite mapping has not been performed for the *B. licheniformis* α-amylase so that explanations of the change in action pattern can only be speculative. A similar change in pattern of oligosaccharide products towards those of lower DP has also been observed when active site tryptophanyl residues of porcine pancreatic and *B. subtilis* α-amylases were modified with 2-hydroxy-5-nitrobenzyl bromide [22].

The ability of the α-amylase to hydrolyse starch, including native starch, in ethanol-aqueous buffer

(7:3, v/v) has been utilized in the preparation of starch-free, cereal endosperm cell walls [23] and may be applied in the isolation of plant cell walls for dietary fibre investigations. In these applications it is essential that α-amylase is free from contaminating polysaccharide hydrolases and contains no carbohydrate. The commercial *B. lichenifórmis* enzyme and similar preparations contain active hydrolases for (1→3)- and (1→3,1→4)-β-glucans but the purified enzyme is free from these contaminants and from associated carbohydrate.

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