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The raw starch-degrading alkaline amylase of *Bacillus* sp IMD 370

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The amylase of *Bacillus* sp IMD 370 is the first report of an alkaline amylase with the ability to digest raw starch. The amylase could degrade raw corn and rice starches more effectively than raw potato starch. It showed no adsorbability to any type of raw starch at any pH value tested. The enzyme digested raw corn starch to glucose, maltose, maltotriose and maltotetraose. The maximum pH for raw starch hydrolysis was pH 8.0 compared to pH 10.0 for soluble starch hydrolysis. The metal chelator, ethylenediaminetetraacetic acid, strongly inhibited raw starch-digestion and its effect was reversed by the addition of divalent cations. Degradation of raw starch was stimulated six-fold in the presence of β-cyclodextrin (17.5 mM).

Keywords: alkaline; amylase; raw starch; β-cyclodextrin

Introduction

In view of energy costs, effective utilisation of natural resources and viscosity problems, direct hydrolysis of starch below the gelatinization temperature is desirable. This need is manifested in the present interest in amylases capable of digesting native starch at moderate temperatures. Although microorganisms reported to be good producers of raw starch-digesting amylases are mostly fungi, there have also been reports of raw starch degradation by bacterial α-amylases [3]. However, there is no published report of an alkaline amylase capable of degrading raw starch granules.

There has always been a correlation between raw starch hydrolysis and raw starch adsorption in fungal enzymes though this is not always the case with bacterial α-amylases [7]. For instance, the raw starch-digesting amylase of *B. subtilis* 65 does not adsorb onto any type of raw starch [7] although an affinity site for raw starch was suggested as α-cyclodextrin (α-CD) was specifically adsorbed onto the enzyme, inhibiting its digestion of raw starch [8]. Complete inhibition of adsorption and digestion by α-CD was also noted for the α-amylase of *Lipomyces starkeyi* HN-606 [13].

This paper deals with the raw starch-degrading properties of the alkaline amylase of alkalophilic *Bacillus* sp IMD 370. This amylase is unique in its ability to degrade raw starch at alkaline pH and in the interaction of this activity with cyclodextrins.

Materials and methods

Materials: Corn starch was obtained from CPC (Ireland). α- and β-Cyclodextrin and potato starch were purchased from Sigma (St Louis, MO, USA). All other chemicals used were Analar Grade reagents.

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Microorganism: *Bacillus* sp IMD 370 was maintained and grown on alkaline medium (pH_i 10.0) as previously described [12].

Enzyme production and purification: The enzyme was produced and purified as previously described [11].

Enzyme assay: α-Amylase was assayed by adding 0.5 ml of enzyme to soluble starch (1%, w/v) in 0.1 M glycine-NaOH buffer, pH 10.0 and incubating the mixture at 40° C for 30 min. The reaction was stopped and the reducing sugars determined using dinitrosalicylic acid according to the method of Bernfeld [2]. An enzyme unit is defined as the amount of enzyme releasing 1 mg glucose equivalents from the substrate, per 30 min at 40° C.

Hydrolysis of raw starch: Raw starch granules were incubated in a shaking water bath at 40° C with enzyme in Tris maleate buffer (0.1 M, pH 8.0). After various time intervals samples were removed from the reaction mixture, boiled for 3 min and centrifuged at 5400 × *g* for 5 min in an MSE bench centrifuge. The reducing sugars in the resulting supernatant phase were determined by the dinitrosalicylic acid method.

Raw starch adsorption: The method used was that of Hayashida and Flor [6].

HPLC analysis of hydrolysates: Identification and quantification of maltooligosaccharides was achieved by HPLC as previously described [4].

Results

Raw starch digestion

The α-amylase of *Bacillus* sp IMD 370 showed a distinct maximum at pH 8.0 for raw starch digestion (Figure 1) compared to pH 10.0 for soluble starch hydrolysis [11]. The ability of the enzyme to degrade native rice, potato and corn starch at pH 8.0 showed 21.7% and 15.8% digestion

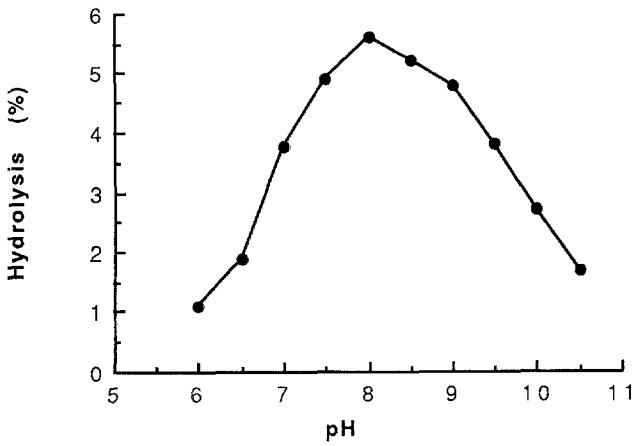

Figure 1 The pH profile of the α-amylase of *Bacillus* sp IMD 370 on raw starch digestion. Hydrolysis was estimated using native corn starch (1%, w/v) as substrate in 0.1 M Universal buffer at the pH values indicated for 3 h at 40° C

of corn and rice starch, respectively after 24 h (Figure 2). However, potato starch proved the most resistant and hydrolysis was limited. Even after 24 h only 3% hydrolysis of potato starch occurred.

End-products formed on raw starch digestion

Only glucose, maltose, maltotriose and maltotetraose were produced on raw corn starch hydrolysis by the alkaline amylase (Table 1). No higher oligosaccharides were detected in the HPLC analysis of the hydrolysates. Glucose (16.4%) and maltose (0.7%) were the final products after enzymic digestion of corn starch for 24 h.

Effect of inhibitors on raw and soluble starch-hydrolysing activities

EDTA completely inhibited both soluble and raw starch-hydrolysing activities. This was effected after 15 min with 3.0 mM EDTA in the case of the raw starch-hydrolysing activity, while 4.5 mM effected complete inactivation of the soluble starch-hydrolysing activity after 10 min (data not shown). After treatment with EDTA the enzyme was dialysed for 12 h and the raw starch-degrading activity of the amylase was totally recovered on the addition of Ba^{2+}, Ca^{2+}, Mg^{2+} and Mn^{2+} (10 mM). The same metals were without effect on the recovery of the soluble starch-hydrolysing activity.

Table 1 Products of *Bacillus* sp IMD 370 α-amylase action on raw corn starch

Time (h)	Sugar (%, w/w)			
	Glucose	Maltose	Maltotriose	Maltotetraose
1	0.3	0.5	2.2	0.2
3	1.2	1.2	1.7	0
12	12.9	1.1	0.9	0
24	16.4	0.7	0	0

Enzyme (2500 units g^{-1} starch) was incubated with corn starch granules at pH 8.0 (0.1 M Tris maleate buffer) at 40° C. End-products were determined by HPLC

Cyclodextrins are known inhibitors of raw starch digestion by α-amylases. α- or β-Cyclodextrins had no effect on soluble starch-hydrolysing activity of the alkaline amylase. Similarly, α-cyclodextrin neither stimulated nor inhibited raw starch digestion while β-cyclodextrin stimulated this activity. Corn starch hydrolysis gradually increased with increasing β-cyclodextrin concentration up to 17.5 mM. No further increase in activity was observed at higher cyclodextrin concentrations (Figure 3). However, a similar stimulatory effect was observed for potato and rice starch hydrolysis. A six-fold increase in digestion of the three raw starches was observed with β-cyclodextrin at 17.5 mM concentration.

Fungal α-amylases and amyloglucosidases have correlated abilities to digest raw starch and to adsorb onto raw starch. The raw starch digesting α-amylase of *Bacillus* sp IMD 370 showed no adsorbability onto any kind of raw starch, at any pH, therefore adsorbability was apparently not necessary for raw starch digestion by this enzyme. The

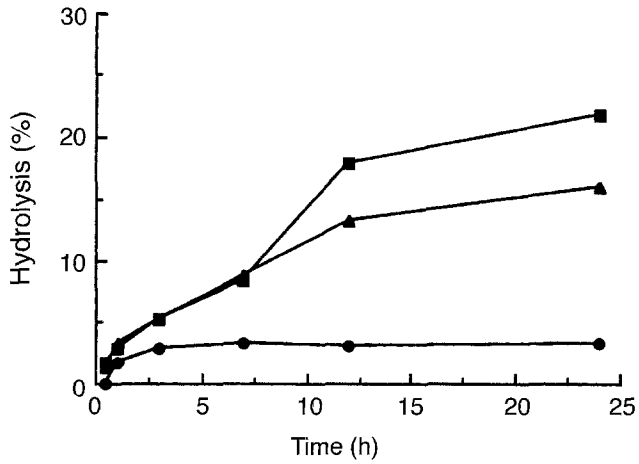

Figure 2 Enzymatic degradation of native starches from potato, corn and rice. Starch was incubated with 10 ml of enzyme in a total volume of 20 ml at pH 8.0 (0.1 M Tris maleate buffer) and sampled over the times indicated. ■, corn starch; ▲, rice starch; ●, potato starch

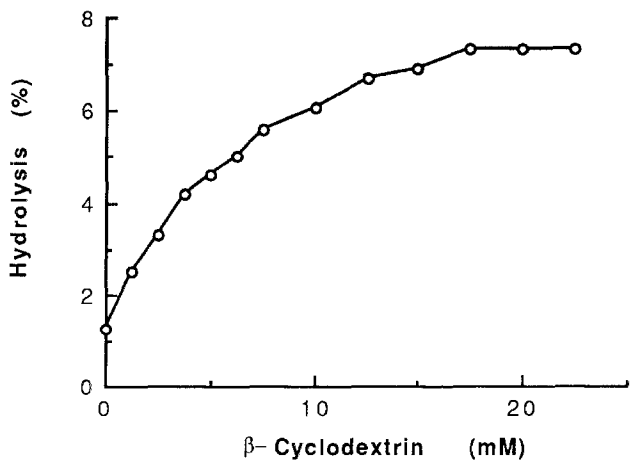

Figure 3 Effect of β-cyclodextrin on raw starch digestion by *Bacillus* sp IMD 370. Percentage hydrolysis was estimated in the presence of β-cyclodextrin at the concentrations indicated, after 3 h at 40° C. The substrate was corn starch (1%, w/v) at pH 8.0 (0.1 M Tris maleate buffer)

enzyme also failed to adsorb onto any of the raw starches (potato, rice or corn) in the presence of β-cyclodextrin. The pH activity profiles for raw corn starch digestion were similar in the presence or absence of β-cyclodextrin with no shift from maximum activity at pH 8.0.

Discussion

The preliminary step in the starch saccharification process involves gelatinisation of starch at high temperature. Due to the industrial application of starch in the food and beverage industry the costliness of the aforementioned step has led to investigations into the enzymatic hydrolysis of raw starch. The traditional role of starch in industry has changed in recent years particularly in relation to its increased application in non-food related industries [10].

The alkaline α-amylase of *Bacillus* sp IMD 370 is similar to other raw starch-degrading enzymes in its capacity to hydrolyse one source of raw starch in preference to another [13,15] and in having a different pH optimum for soluble and raw starch hydrolysis [14]. The finding that metal chlorides restored raw starch-digesting activity of the alkaline amylase after treatment with EDTA with no effect on soluble starch-hydrolysing activity has also been reported for the raw starch-digesting amylase of *Bacillus* sp B1018 [9]. It suggests that the conformation of the site responsible for raw starch digestion has not been interfered with or has been retained and the resultant addition of metal reactivates the system.

However, in other areas the alkaline enzyme is quite different from any reported to-date. The existence of a raw starch affinity site separate from the active site is not as well established for bacterial α-amylases as it is for fungi. However, bacterial α-amylases generally adsorb to the substrate with the exception of *Bacillus subtilis* 65 α-amylase [7]. The ability of the latter to digest raw starch and adsorb on to α-CD-Sepharose 6B was simultaneously lost when a specific domain corresponding to a raw starch affinity site was deleted by proteolysis [8]. α-CD showed specific inhibition of raw corn starch digestion by *B. subtilis* 65 α-amylase by complexing with the affinity site apart from the catalytic site on the enzyme molecule [8]. Similarly, competitive adsorption between CDs and raw starch granules occurred at the affinity site of *Aspergillus awamori* var *kawachi* glucoamylase [5] inhibiting raw starch digestion. Belshaw and Williamson [1] proved that β-CD binds to the binding domain of glucoamylase of *Aspergillus niger* at the same site(s) as raw starch, competitively inhibiting adsorption. The catalytic domain showed no interaction with β-CD. However, the raw starch-digesting amylase of *Bacillus* sp IMD 370 contrasts sharply with the previously described systems as α-CD has no effect on raw starch digestion and β-CD gave a six-fold increase in rice, potato and corn starch degradation. This enzyme does not adsorb on raw starch so whether there is a separate raw starch affinity site or not is not immediately evident. Similarly, it is not clear how β-CD effects a six-fold increase in raw starch digestion but the cyclic molecule must in some way facilitate the formation of a productive complex between the enzyme and raw starch.

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