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Raw starch degradation by the non-raw starch-adsorbing bacterial alpha amylase of *Bacillus* sp. IMD 434

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Abstract

The raw starch-digesting alpha amylase of *Bacillus* sp. IMD 434 was purified to homogeneity and displayed substantial hydrolysis of raw starch but did not adsorb onto the insoluble substrates, corn, rice, wheat or potato starch, at any of the pH values examined. The degree of hydrolysis ranged from 10% hydrolysis of potato starch to 32% hydrolysis of corn starch after 24 h. α- and β-Cyclodextrins (CDs) inhibited raw starch digestion but did not affect hydrolysis of soluble starch. In the presence of 10 mM α-CD or β-CD, hydrolysis of raw corn starch by the amylase decreased by 88 and 97%, respectively. The enzyme did adsorb onto α-CD Sepharose 6B, suggesting that an affinity site may be present on this non-raw starch-adsorbing amylase. After incubation with Pronase E, alpha amylase (434a M_r 69,200) was hydrolysed into two components, a large enzymatically active component, EA 434b (M_r 56,200) and a small inactive peptide, IA 434c (M_r 13,000). EA 434b, although active on soluble starch, was incapable of hydrolysing raw starch, unable to adsorb onto raw starch and lost its ability to adsorb onto α-CD Sepharose 6B. Conversely, IA 434c was inactive on soluble and raw starch, did not adsorb onto raw starch but did adsorb onto α-CD Sepharose 6B and a range of linear maltooligosaccharide Sepharose 6B matrices. Thus, the alpha amylase simultaneously lost the ability to hydrolyse raw starch and adsorb onto α-CD Sepharose 6B when IA 434c was removed by proteolysis. The ability of the raw starch-digesting alpha amylase to adsorb onto α-CD Sepharose 6B was then exploited successfully in the development of a one-step purification for the amylase using CD affinity chromatography.

Keywords: Alpha amylase; Raw starch-digesting; Non-raw starch-adsorbing; Cyclodextrin affinity chromatography

1. Introduction

With fungal amyloglucosidases and most bacterial alpha amylases there is a strong correlation between raw starch digestion and raw starch adsorption. Adsorption of these enzymes onto the raw starch granule is essential for raw starch hydrolysis and the ability to adsorb onto the insoluble substrate has been attributed to the presence of a raw starch-binding domain [1–4]. However, Hayashida et al. [5] reported that the alpha amylase of *Bacillus subtilis* 65 could hydrolyse raw starch although it showed no adsorption onto raw starch. An affinity site for raw starch was suggested for this enzyme since α-cyclodextrin (α-CD) specifically adsorbed onto the enzyme, inhibiting raw starch digestion. It is of interest to note that this bacterial alpha amylase can be mentioned [6–8] as an exception to the more widely held view that all raw starch-di-

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gesting enzymes possessing affinity sites adsorb onto raw starch.

This paper deals with the raw starch-digesting alpha amylase of *Bacillus* sp. IMD 434. Unlike most bacterial alpha amylases [3,7,9,10] this enzyme does not adsorb onto raw starch. Interestingly, its raw starch digestion is only inhibited by CDs. It appears that CDs inhibit raw starch digestion of this enzyme by interacting with its affinity site. Given that affinity chromatography is an extremely effective purification technique, the ability of the enzyme to bind onto α-CD Sepharose 6B was successfully exploited in the development of a one-step purification protocol.

2. Results

The alpha amylase of *Bacillus* sp. IMD 434 displayed optimum hydrolysis of raw starch at pH 6.0. Corn, rice and wheat starch were the most susceptible to amylase attack, being degraded by 32, 28 and 18%, respectively, after 24 h (Fig. 1). Potato starch was the least susceptible to amylase attack as only 10% hydrolysis was observed after 24 h. The alpha amylase did not adsorb onto any of the raw starches examined (corn, rice, wheat, potato). Varying the substrate concentration (0.5, 1, 2 and 10% (w/v)), the temperature of incubation (4, 22 and 40 °C), the pH (4, 5, 6, 7, 8 and 9) and the incubation times (15 min, 1 and 3 h) had no effect on the ability of the enzyme to adsorb onto raw starch. The enzyme hydrolysed untreated and acid-treated starches to the same extent, hydrolysing corn starch and potato starch to 38 and 10%, respectively and, the acid-treated starches to 39 and 10%, respectively, after 24 h. No adsorption onto the acid-treated starches was observed.

Although α-CD and β-CD had no effect on the hydrolysis of soluble starch, raw starch digestion was inhibited (Table 1). The lack of inhibition of soluble starch hydrolysis indicates that the active site of the enzyme is not

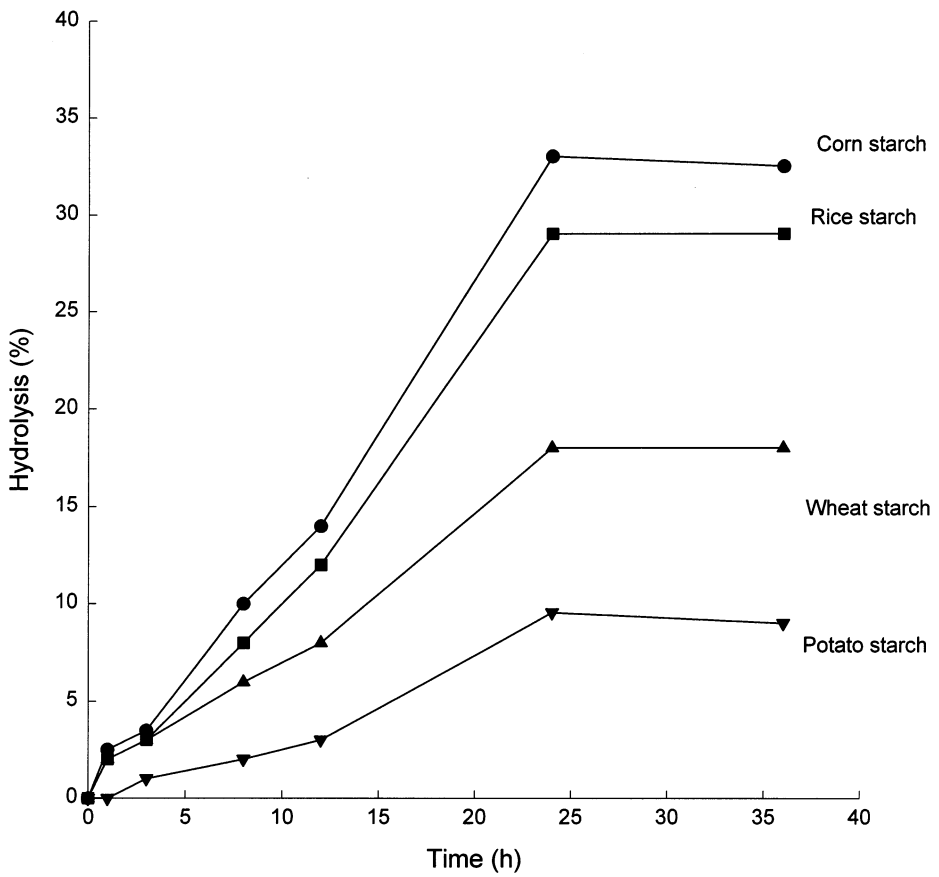

Fig. 1. Enzymatic degradation of native starches from corn, rice, wheat and potato by the alpha amylase of *Bacillus* sp. IMD 434. Starch was incubated with enzyme (1000 U/g) at pH 6 (0.1 M citrate buffer) at 40 C° and sampled at the times indicated.

Table 1
Effect of cyclodextrins on starch hydrolysis by the alpha amylase of *Bacillus* sp. IMD 434

Cyclodextrin	(mM)	Relative activity (%)	
		Soluble starch[a]	Raw starch[b]
α-CD	0	100	100
	1	100	25
	10	96	12
β-CD	1	100	20
	10	95	3

[a] The enzyme was assayed on starch (1%, w/v) in 0.1 M phosphate buffer, pH 6.0 for 30 min at 40 °C and this, the control, was set as 100% activity. Enzyme was preincubated with cyclodextrin, at the concentrations indicated, for 15 min at 20 °C, prior to alpha amylase activity being determined as above and the relative activity expressed as a percentage of the control.

[b] The degree of hydrolysis obtained after incubating the enzyme (1000 U/g starch) with raw corn starch (1%, w/v) in 0.1 M citrate buffer, pH 6.0 for 6 h was used as the control and set as 100%. Enzyme was preincubated with cyclodextrin, at the concentrations indicated, for 15 min at 20 °C prior to the addition of corn starch (1%, w/v) in 0.1 M citrate buffer, pH 6.0. The reaction mixture was incubated at 40 °C and the activity determined after 6 h was expressed as a percentage of the control.

affected by the CDs. The possibility that these cyclical maltooligosaccharides were interfering with an adsorption site was investigated. A total of 85–88% of the alpha amylase of *Bacillus* sp. IMD 434 adsorbed onto α-CD Sepharose 6B. The fact that the enzyme adsorbed onto CDs suggests that it may contain an affinity site. Thus, the inhibition of raw starch digestion, in the presence of CDs, may be caused by the CDs competing with the raw starch granules for the affinity site on the enzyme.

The purified enzyme, 434a (M_r 69,200) was incubated with Pronase E and the fragments in the digest were analysed by SDS-PAGE and fractionated by gel filtration (Figs. 2 and 3). The results of SDS-PAGE (Fig. 2) clearly indicate that the original peptide was hydrolysed by Pronase treatment into two units of smaller size. The Pronase-digested alpha amylase yielded three main fractions after gel filtration: Fractions 1, 2 and 3 (Fig. 3). Fractions 1 and 2 were enzmatically active on soluble starch whereas Fraction 3 was inactive. Fraction 1, the first eluted protein, was designated as non-digested alpha amylase, 434a, as the relative molecular mass correlated with that determined earlier for the original enzyme. However, only a small quantity of non-digested enzyme remained as the majority of the protein had been hydrolysed into the two smaller units. The larger of these units (M_r 56,200), Fraction 2, retained activity on soluble starch and was designated EA 434b. The smaller hydrolysed unit (M_r 13,000) was eluted in Fraction 3 and was an inactive peptide (IA 434c).

The properties of EA 434b were examined with respect to its ability to hydrolyse raw starch and to adsorb onto both raw starch and α-CD Sepharose 6B. EA 434b lost the ability to degrade raw starch. As with the original alpha amylase no adsorption onto raw starch was detected. However, unlike the non-digested alpha amylase of *Bacillus* sp. IMD 434, which adsorbed onto the α-CD Sepharose 6B, the hydrolysed enzyme had significantly reduced ability to bind to this complex. A total of 88% of the non-hydrolysed alpha amylase effectively adsorbed onto α-CD

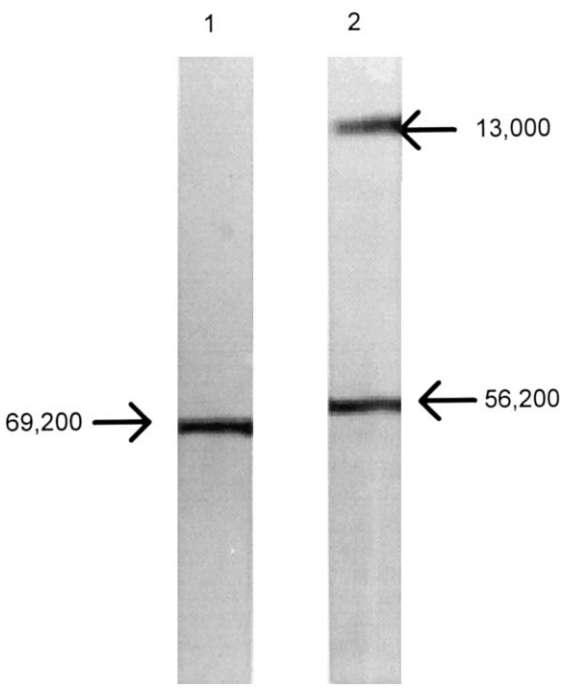

Fig. 2. SDS-PAGE of the original alpha amylase and the Pronase hydrolysed alpha amylase of *Bacillus* sp. IMD 434. Lane 1, the original alpha amylase; lane 2, the hydrolysed amylase. Pronase hydrolysis was carried out as stated in Section 4.

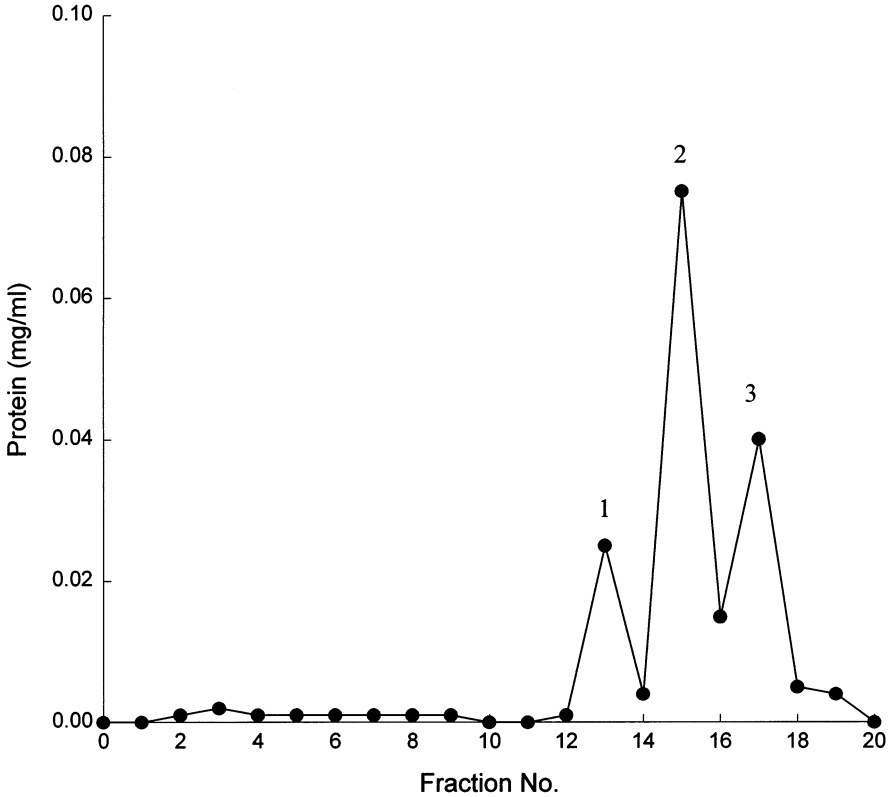

Fig. 3. Gel filtration of the proteinase treated alpha amylase of *Bacillus* sp. IMD 434. Peaks 1, 2 and 3 indicate non-hydrolsed amylase (434a), hydrolysed component (EA 434b) and the small inactive peptide (IA 434c), respectively.

Sepharose 6B but the adsorption of EA 434b decreased to 10%. This result indicates that the alpha amylase simultaneously lost raw starch digestibility and α-CD Sepharose 6B adsorbability when the smaller peptide was removed by proteolysis.

IA 434c was inactive on soluble starch, did not adsorb onto raw starch but adsorbed onto α-CD Sepharose 6B. Since IA 434c bound cyclic maltooligosaccharides, its ability to bind linear maltooligosaccharides was investigated. The linear maltooligosaccharides maltose, maltotetraose, maltohexaose and maltoheptaose were bound to an insoluble support, Sepharose 6B, and it was found that between 60 and 70% of IA 434c adsorbed to the matrices. In comparable experiments using bovine serum albumin no adsorption was detected, indicating that adsorption onto linear maltooligosaccharides is a specific property of the peptide, IA 434c. The ability of IA 434c to adsorb onto α-CD Sepharose 6B and the linear maltooligosaccharides Sepharose 6B matrices confirms its role as an affinity site on the alpha amylase of *Bacillus* sp. IMD 434.

On finding that the alpha amylase of *Bacillus* sp. IMD 434 possessed an affinity site, the purification protocol was redesigned to exploit this property. The crude CFS was applied directly onto the affinity column and eluted with buffer containing α-CD. This single step yielded a highly purified protein that had good specific activity (2025 U/mg protein) and excellent recovery (65%) (Table 2). The homogeneity of the enzyme was confirmed by SDS-PAGE.

3. Discussion

The alpha amylase of *Bacillus* sp. IMD 434 was capable of hydrolysing raw starch. Corn, rice and wheat starch were most susceptible to amylase attack with potato starch the most resistant. However, regardless of the starch examined, the alpha amylase of *Bacillus* sp. IMD 434 did not adsorb onto raw starch. This is contrary to the reports on most bacterial amylases where raw starch adsorption is essential for raw starch digestion [4,7,9–11]

and the ability to adsorb onto raw starch has been attributed to a specific binding site present on the enzyme [4,7]. The raw starch-digesting alpha amylase of *B. subtilis* 65 is an exception to the above group of enzymes. Hayashida et al. [5] showed that although an affinity site is present, this enzyme did not adsorb onto raw starch. Likewise, the work here highlights another raw starch-digesting but non-raw starch-adsorbing bacterial alpha amylase. After proteinase treatment the alpha amylase of *Bacillus* sp. IMD 434 is hydrolysed into two components. The larger protein remained active on soluble starch but lost the ability to hydrolyse raw starch and adsorb onto α-CD Sepharose 6B, while the smaller peptide was enzymatically inactive but was able to adsorb onto α-CD Sepharose 6B and linear maltooligosaccharides bound to the Sepharose 6B matrix. Therefore, this bacterial alpha amylase also possesses an affinity site which is essential for hydrolysis of raw starch as its subsequent removal results in a loss in raw starch-digestion. It thus appears that two types of raw starch-digesting bacterial alpha amylases exist. The first type resemble the well-studied fungal amyloglucosidases and are raw starch-digesting and raw starch-adsorbing. They include the alpha amylases of *B. circulans* F2 and *Clostridium butyricum* [4,9]. The discovery of the alpha amylase of *Bacillus* sp. IMD 434 shows that the alpha amylase of *B. subtilis* 65 [5] is not unique among raw starch-digesting alpha amylases. It is more likely that these two enzyme systems belong to a second group, which are raw starch-digesting but non-raw starch-adsorbing alpha amylases. Although the latter do not adsorb onto raw starch, the presence of affinity sites on these amylases suggest that some interaction must occur between these raw starch-digesting but non-raw starch-adsorbing alpha amylases and the insoluble substrate.

CD affinity chromatography proved highly effective in purifying the enzyme in one-step. Compared with the previously employed purification protocol which involved acetone precipitation, ion-exchange chromatography and hydrophobic interaction chromatography this method was more efficient, yielded an enzyme preparation with better specific activity and purification fold and, more significantly the enzyme recovery was far superior. Traditionally though, the role of CD affinity chromatography in the purification of amylolytic enzymes has been based on the specific interaction that occurs between the active sites of enzymes and the CDs. For the latter this interaction has been directly attributed to the fact that CDs are competitive inhibitors of some amylases [12–14]. Here, however, the interaction with the CDs was shown to occur at the affinity site of the enzyme. Similarly, with other raw starch-digesting amylases the inhibition of raw starch hydrolysis by CDs has been shown to be due to competition for the affinity site on such enzymes [15,16]. An important property of CD affinity chromatography, the method proposed here for the purification of raw starch-digesting amylases, is that it can be used for the purification of both raw starch-adsorbing and non-raw starch-adsorbing, raw starch-digesting amylases.

4. Experimental

Materials.—Corn starch was obtained from CPC (Ireland). α- and β-CD, potato starch and Pronase E were purchased from Sigma (St. Louis, MO). Rice and wheat starch were purchased from BDH. Epoxy-activated Sepharose 6B was purchased from Pharmacia Fine Chemicals. All other chemicals used were Analar grade reagents.

Table 2
One-step purification of the alpha amylase of *Bacillus* sp. IMD 434

Stage	Specific activity (U/mg protein)	Purification (*x*-fold)	Recovery (%)
CFS	5.4	1.0	100
α-CD Sepharose 6B affinity chromatography[a]	2025	375	65

[a] The column was equilibrated with 0.05 M citrate buffer, pH 6.0 and the enzyme was eluted with 0.05 M citrate buffer, pH 6.0 containing 10 mg/mL α-CD.

Microorganisms.—*Bacillus* sp. IMD 434 was isolated from a screening programme in this laboratory and was maintained at room temperature on 1% starch nutrient agar slopes (pH 7.0).

Enzyme production and purification.—A standard inoculum medium was used (g/L): soluble starch 10.0, bactopeptone 10.0, yeast extract 2.0, KH_2PO_4 0.05, $MnCl_2 \cdot 4\ H_2O$ 0.0015, $MgSO_4 \cdot 7\ H_2O$ 0.25, $CaCl_2 \cdot 2\ H_2O$ 0.05, $FeSO_4 \cdot 7\ H_2O$ 0.01, pH 7.0. After 9 h at 40 °C, a 1% (v/v) inoculum was transferred into 250 mL Erlenmeyer flasks containing 50 mL production medium. The production medium was (g/L): soluble starch 30.0, corn steep liquor 30.0, KH_2PO_4 0.05, $MnCl_2 \cdot 4\ H_2O$ 0.0015, $MgSO_4 \cdot 7\ H_2O$ 0.25, $CaCl_2 \cdot 2\ H_2O$ 0.05, $FeSO_4 \cdot 7\ H_2O$ 0.01, pH 7.0. The cells were shaken at 200 rpm in a Gallenkamp incubator at 40 °C. Cells were removed from the medium by centrifugation at 16,300g for 20 min at 4 °C in a Sorvall RC2-B centrifuge and enzyme activity in the cell-free supernatant (CFS) was measured.

Cyclodextrin affinity chromatography.—A modification of the method of Vretblad [12] was used to prepare the α-CD Sepharose 6B gel in that the incubation was carried out at 40 °C. The prepared gel was then poured into a Pharmacia column ($7 \times 60\ mm^2$) and packed under pressure using a Pharmacia P-1 pump with 0.05 M citrate buffer, pH 6.0 with a flow rate of 0.4 mL/min. Enzyme was applied in 0.05 M citrate buffer, pH 6.0 and eluted from the column with the same buffer containing 10.0 mg/mL α-CD using a flow rate of 0.2 mL/min.

Enzyme assay.—Alpha amylase was assayed by adding 0.5 mL of enzyme to 0.5 mL soluble starch (1%, w/v) in 0.1 M phosphate buffer, pH 6.0, for 30 min at 40 °C. The reaction was stopped and the reducing sugars determined with dinitrosalicylic acid according to the method of Bernfeld [17]. An enzyme unit is defined as the amount of enzyme releasing 1 mg of glucose equivalents from the substrate per 30 min at 40 °C.

Determination of protein.—Protein was determined using a UV spectrophotometer (LKB, Ultrospec II) taking readings at 280 nm. Water or buffer was used as a blank. A standard curve for the conversion of OD readings to mg protein was obtained using a series of dilutions of bovine serum albumin.

Hydrolysis of raw starch.—Raw starch granules (100 mg) were incubated in a shaking water bath at 40 °C with 5.0 mL enzyme (1000 U/g starch) in 0.1 M citrate buffer (5.0 mL), pH 6.0 containing a few drops of toluene to prevent microbial contamination. After various time intervals samples were removed from the reaction mixture, boiled for 3 min and centrifuged at 3000*g* for 5 min in an MSE bench centrifuge. The reducing sugars in the resulting supernatant were determined by the dinitrosalicylic acid method.

Raw starch adsorption.—The method used was that of Hayashida and Flor [18]. Enzyme (1000 U/g starch) was added to 0.1 M citrate buffer pH 6.0 and 100 mg raw starch was added to 10 mL of the prepared enzyme solution and incubated with constant shaking at 4, 20 and 40 °C for various time periods. After centrifugation at 3000*g* for 5 min, the alpha amylase activity of the supernatant was assayed and compared with that of the original alpha amylase solution. The adsorption rate (AR) was defined by the following equation: $AR\ (\%) = [(B - A)/B]100$ where (A) indicates the residual amylase activity after the adsorption test and (B) that of the original enzyme solution.

Acid hydrolysis of raw starch.—Raw corn and potato starches were incubated in 0.1 N HCl at 25 °C in a shaking water bath for 24 h. The starches were then washed thoroughly with distilled water and equilibrated in 0.1 M citrate buffer, pH 6.0. Enzymatic hydrolysis of the acid-treated starches was carried out in the usual manner.

Adsorption onto α-CD Sepharose 6B.—α-CD Sepharose 6B was prepared as described above. Equal volumes of enzyme solution (1000 U/g starch) and equilibrated gel were mixed and incubated at 40 °C for 15 min with constant shaking. After centrifugation at 3000*g* for 5 min, the alpha amylase activity in the supernatant was determined and the amount of enzyme adsorbed calculated using the equation for raw starch adsorption. Control gels (minus α-CD) were also incubated with enzyme.

Proteolytic digestion of alpha amylases with Pronase E.—*Bacillus* sp. IMD 434 alpha amylase (50 mg) was incubated in a shaking water bath with 10 mg of Pronase E in 10 mL of 0.05 M Tris–HCl buffer, pH 7.8 at 40 °C for 16 h. After incubation, the reaction mixture was centrifuged at 3000*g* in a bench centrifuge, equilibrated in 0.05 M phosphate buffer, pH 7.5 containing 0.15 M NaCl and fractionated on Superose 12 column (300 × 10 mm^2) previously equilibrated with the same buffer. Fractions having a 1 mL volume were collected at a flow rate of 0.5 mL/min. The fractions containing soluble starch hydrolysing activity were desalted by dialysis and designated according to their M_r as either non-digested alpha amylase or hydrolysed enzyme. Similarly a low molecular weight protein was collected, desalted and designated as the possible raw starch affinity site.

SDS-PAGE.—A total of 40 μg of protein was applied to SDS-PAGE which was carried out according to the Phast System Separation Technique File no. 110 with silver staining.

Determination of adsorption to Gn–Sepharose 6B.—The non-cyclic sugars G2, G4, G6 and G7 were coupled to Sepharose 6B in the same manner as α-CD. The adsorption of the raw starch affinity site of the alpha amylase from *Bacillus* sp. IMD 434 onto the gels was measured by incubating the protein (5 mg) with an equal volume of the gel and then measuring the $A_{280.}$ The adsorption rate was calculated as described for raw starch. To determine if it was specifically the sugars that the affinity sites were adsorbing to, controls were carried out by incubating the proteins with gels that had no sugars bound but the available ligand sites had been blocked with ethanolamine.

References

[1] S. Hayashida, K. Nakahara, K. Kuroda, T. Miyata, S. Iwanaga, *Agric. Biol. Chem.*, 53 (1989) 135–141.
[2] Y. Hayashida, K. Nakahara, W. Kanlayakrit, T. Hara, Y. Teramoto, *Agric. Biol. Chem.*, 53 (1989) 143–149.
[3] C.H. Kim, G.W. Zhang, Y. Maruyama, H. Taniguchi, *Agric. Biol. Chem.*, 54 (1990) 2767–2768.
[4] C.H. Kim, H. Sata, H. Taniguchi, D.S. Lee, *Biochim. Biophys. Acta*, 1122 (1992) 243–250.
[5] S. Hayashida, Y. Teramoto, T. Inoue, S. Mitsuiki, *Appl. Environ. Microbiol.*, 56 (1990) 2584–2586.
[6] W.J. Wang, A.D. Powell, C.G. Oates, *Carbohydr. Polym.*, 26 (1995) 91–97.
[7] P. Itkor, N. Tsukagoshi, S. Udaka, *Biochem. Biophys. Res. Commun.*, 166 (1990) 630–636.
[8] K. Fukuda, Y. Teramoto, M. Goto, J. Sakamoto, S. Mitsuiki, S. Hayashida, *Biosci. Biotech. Biochem.*, 56 (1992) 556–559.
[9] T. Tanaka, E. Ishimoto, Y. Shimomura, M. Taniguchi and, S. Oi, *Agri. Biol. Chem.*, 51 (1987) 399–405.
[10] D.J. Wibenga, G. Beldman, A. Veen, D.J. Binnema, *Appl. Microbiol. Biotechnol.*, 35 (1991) 180–184.
[11] C.L. Jeang, Y.-L. Lee, L.-W. Lee, *Biochem. Mol. Biol. Int.*, 35 (1995) 549–557.
[12] P. Vretblad, *FEBS Lett.*, 47 (1974) 86–89.
[13] E. Bourke, W.M. Fogarty, *Soc. Gen. Microbiol. Proc.*, 2 (1975) 80–81.
[14] A. Viksø-Nielsen, T. Christensen, M. Bojko, J. Marcussen, *Physio. Plantarum*, 99 (1997) 190–196.
[15] N.J. Belshaw, G. Williamson, *Biochim. Biophys. Acta*, 1078 (1991) 117–120.
[16] K Fukuda, Y. Teramoto, M. Goto, J. Sakamoto, S. Mitsuiki, S. Hayashida, *Biosci. Biotech. Biochem.*, 56 (1992) 556–559.
[17] P. Bernfeld, *Methods Enzymol.*, 1 (1955) 149–158.
[18] S. Hayashida, P.Q. Flor, *Agric. Biol. Chem.*, 45 (1981) 2675–2681.