Active-site- and substrate-specificity of *Thermoanaerobium* Tok6-B1 pullulanase

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Thermoanaerobium Tok6-B1 pullulanase (EC 3.2.1.41) was active on α 1–6-glucosidic linkages of pullulan, amylopectin and glycogen and the α 1–4 linkages of amylose, amylopectin and glycogen but not of pullulan. Hydrolysis of short-chain-length malto-oligosaccharides (seven or fewer glucose residues) yielded maltose as product. Pullulan hydrolysis was pH-dependent and a plot of $\log(V/K_m)$ versus pH implied a carboxy group with pK_a 4.3 at the active site. Modification with 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide (EDAC) confirmed this view, and analysis of the order of reaction and inactivation kinetics suggested the presence of a single carboxy group at a catalytic centre of the active site. EDAC-mediated inhibition of pullulan α 1–6-bond hydrolysis was relieved by amylose or pullulan. Similarly both pullulan and amylose protected the activity directed at α 1–4 bonds of amylose from EDAC inhibition. When both amylose and pullulan were simultaneously present, the observed rate of product formation closely fitted a kinetic model in which both substrates were hydrolysed at the same active site.

INTRODUCTION

Pullulan is a linear glucan of maltotriosyl units (Glc α 1–4Glc α 1–4Glc) linked through α 1–6-glucosidic linkages. The α 1–6 linkages are considered partially to mimic the α 1–6 branch points of amylopectin, and pullulan has been widely employed as a model substrate for starch-debranching enzymes [1–3].

Enzymes that hydrolyse pullulan have been classified into four types [4]: (a) pullulanase (EC 3.2.1.41), hydrolysing α 1–6 linkages of pullulan to produce maltotriose, (b) isopullulanase (EC 3.2.1.57), hydrolysing α 1–4 linkages of pullulan to produce isopanose (Glc α 1–4Glc α 1–6Glc), (c) an α -amylase (EC 3.2.1.1) from *Thermoactinomyces vulgaris*, primarily acting on α 1–4 linkages of pullulan to produce panose (Glc α 1–6Glc α 1–4Glc), and (d) glucoamylase (EC 3.2.1.3), sequentially hydrolysing pullulan from the non-reducing terminal to produce glucose.

Thermoanaerobium Tok6-B1 is an extremely thermophilic anaerobic bacterium isolated from a New Zealand geothermal environment [5]. When grown on starch-containing media the organism elaborates a pullulanase [6] that has been isolated and found to be active not only on the α 1–6 linkages of pullulan but also on α 1–4-glucosidic linkages in starch, amylose and amylopectin [7]. ‘True’ pullulanase activity is restricted to α 1–6-glucosidic linkages in pullulan and certain α 1–6-glucosidic-branch-point glucans, e.g. starch [8], whereas isopullulanase and *Thermoactinomyces vulgaris* α -amylase hydrolyse α 1–4 linkages in both pullulan and glucan. Unlike glucoamylase, which is active on both α 1–4 and α 1–6 linkages in pullulan, the *Thermoanaerobium* enzyme will not hydrolyse α 1–4 bonds in pullulan and produces maltotriose rather than glucose as product.

The action of *Thermoanaerobium* Tok6-B1 pullulanase therefore appears to be unique, and the present study

was undertaken to determine if this dual specificity towards α 1–6 bonds of pullulan and α 1–4 linkages of other glucans resides at a single active centre of the enzyme and to investigate its mode of action on various oligosaccharides and polysaccharides.

EXPERIMENTAL

Materials

Thermoanaerobium Tok6-B1 pullulanase was purified over 1600-fold as previously described [7]. Specific activity was 1.79 mmol of reducing sugar product/min per mg of protein, and a 0.5 μ g/ml aqueous solution of the enzyme was stored at -18°C . *Enterobacter aerogenes* pullulanase, EDAC, amylopectins, glycogens, potato amylose, Lintner’s starch, pullulan, cyclomaltohexaose, cyclomaltoheptaose, malto-oligosaccharides, *N*-ethylmaleimide, *p*-chloromercuribenzoate and iodoacetic acid were obtained from Sigma Chemical Co. β -Amylase was purchased from Calbiochem, bacterial α -amylase from BDH Chemicals, short-chain amylose from Aldrich, phenethylamine from Hopkin and Williams and cyclomalto-octaose from Fluka. All other chemicals were commercially available analytical grade.

Enzyme assays

Unless stated otherwise, assays were performed at 70°C with buffers adjusted at 70°C with the use of a temperature-compensated Pye pH electrode. *Thermoanaerobium* pullulanase and α -amylase activities were determined as previously described [7] in 1 mM- CaCl_2 /0.1 M-Mes adjusted to pH 5.6 with 3.0 M-NaOH, with pullulan and amylose respectively as substrates. Commercial α -amylase and β -amylase were assayed at 25°C in 1 mM- CaCl_2 /0.1 M-Mes adjusted to pH 5.6 at 25°C . Reducing-sugar products were determined with *p*-

Abbreviations used: EDAC, 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride; Glc2, Glc3 etc., Glc α 1–4Glc, Glc α 1–4Glc α 1–4Glc etc.; MT2, MT3 etc., Glc α 1–4Glc α 1–4Glc α 1–6Glc α 1–4Glc α 1–4Glc, Glc α 1–4Glc α 1–4Glc α 1–6Glc α 1–4Glc α 1–4Glc α 1–6Glc α 1–4Glc α 1–4Glc etc.

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hydroxybenzoic acid hydrazide [9], with maltotriose as standard.

Enzymic hydrolysis of amylose was also monitored by I_2/KI staining. Reaction mixtures contained either 15 μg of commercial α -amylase, 64 μg of β -amylase, or 0.5 μg of *Thermoanaerobium* enzyme in 2.4 ml of the appropriate buffer containing 1.2 mg of short-chain amylose. After incubation at the appropriate temperature, 0.1 ml of reaction mixture was added to 0.1 ml of 0.1 M-HCl followed by 0.2 ml of 1.0% (w/v) KI + 0.3% (w/v) I_2 in water and 0.5 ml of water. Activity is quoted as percentage change in absorbance at 615 nm compared with zero-time value.

EDAC modification

EDAC modification was performed at 35 °C by dissolving known quantities of EDAC in 25 mM-phenethylamine made up in 0.1 M-Mes adjusted to pH 5.7 at 35 °C. Enzyme solutions were dialysed against buffer before use. Reactions were initiated by adding enzyme and terminated by withdrawing portions and diluting 20-fold with water. Residual pullulanase and α -amylase activities were determined as described above.

Oligosaccharide analysis

Products of glucan hydrolysis were analysed by h.p.l.c. on a Bio-Rad HPX-42A column at 90 °C with sterile filtered Milli-Q water as solvent. Reference standards were commercial malto-oligosaccharides, Glc2 to Glc7 and a partial pullulan hydrolysate of *E. aerogenes* pullulanase containing MT series oligosaccharides (see below).

Preparation of MT-series oligosaccharides

A 60-unit portion of *E. aerogenes* pullulanase was incubated at 30 °C for 80 min in 30 ml of 4% (w/v) pullulan in 0.1 M-sodium acetate adjusted to pH 6.0 with acetic acid. Reaction was terminated by suspending the vessel in boiling water for 5 min and concentrated to 4.5 ml by rotary film evaporation. A 0.5 ml portion of the concentrate containing MT series oligosaccharides [8] was retained as an h.p.l.c. standard, and the remainder was passed through a 47 cm $\times$ 2.6 cm column of Merck Fractogel TSK HW-40(S) equilibrated with water at 0.8 ml/min. Fractions (1.5 ml) were collected and analysed by h.p.l.c., and those containing MT2 alone were pooled and evaporated to dryness.

RESULTS

EDAC modification of pullulanase

The kinetic parameter V/K_m for *Thermoanaerobium* pullulanase showed pH-dependence, and inflexions in the plot of $\log(V/K_m)$ against pH (Fig. 1) suggested the involvement of amino acids with pK_a values of 4.3 and 7.1. It was considered most likely that the pK_a value of 4.3 represented the ionization of a glutamate or aspartate carboxy group, and verification of this was sought by carboxy-group-specific chemical modification with EDAC.

Although carbodi-imides primarily modify carboxy groups [10], in certain circumstances they can react with hydroxy groups of tyrosine or thiol groups of cysteine [11,12]. EDAC-mediated modification of thiol groups was discounted, as the thiol-active reagents 3.5 mM-N-ethylmaleimide, 3.5 mM-p-chloromercuribenzoate and

Fig. 1. Effect of pH on V/K_m for hydrolysis of pullulan by *Thermoanaerobium* pullulanase

K_m and V for pullulan were determined in 100 mM-Mes (●) or 100 mM-Mops (○) buffer containing 1 mM- CaCl_2 .

Fig. 2. Kinetics of inactivation of *Thermoanaerobium* pullulanase by EDAC

Enzyme (final concn. 0.17 $\mu\text{g}/\text{ml}$) was incubated in buffer containing 25 mM-phenethylamine and EDAC at 0.6 mM (○), 1.6 mM (●), 3.2 mM (□), 7.8 mM (■) or 15.4 mM (△). At time intervals samples were withdrawn and diluted 20-fold with water and residual activity was determined. The inset shows the apparent order of reaction with respect to EDAC concentration. Observed pseudo-first-order rate constants (k'_0) were calculated from the slopes in the main Figure.

1.4 mM-iodoacetic acid had no effect on enzyme activity. Reaction of tyrosine with EDAC is reversed by treatment with hydroxylamine, pH 7.0; however activity was not restored to EDAC-modified pullulanase after dialysis for 30 h against 0.4 M-hydroxylamine.

Fig. 3. Double-reciprocal plot for native and EDAC-modified *Thermoanaerobium pullulanase*

Enzyme (final concn. 0.17 $\mu\text{g/ml}$) was incubated in buffer containing 25 mM-phenethylamine and 8 mM-EDAC until residual activity was 14% (○), 30% (●) or 58% (□) of control activity (■) and K_m and V were determined after diluting 20-fold with water.

EDAC-mediated inactivation was not reversed by exhaustive dialysis against water and was therefore due to irreversible reagent modification rather than reagent inhibition. Inactivation of pullulanase with EDAC was both time- and concentration-dependent, and plots of $\log(\text{residual activity})$ against time were linear down to 25% of the control, enabling the pseudo-first-order rate constant k'_0 to be determined from the slope for each EDAC concentration (Fig. 2). The order of reaction was determined from logarithmic plots of k'_0 against EDAC concentration [13,14], yielding a slope of 0.78 (Fig. 2 inset), suggesting that EDAC reacts with a single carboxy group.

The apparent K_m of EDAC-modified pullulanase was identical with that of the native enzyme (Fig. 3), whereas V decreased in line with increased inactivation by the carbodi-imide. The results suggest that the single carboxy group modified by EDAC is at a catalytic centre of the active site but is not involved in substrate binding.

Evidence that pullulanase and α -amylase activities share the same active centre

When enzyme was modified with EDAC in the presence of pullulan or amylose and residual pullulanase (hydrolysis of α 1-6-glucosidic bonds) or α -amylase (hydrolysis of α 1-4-glucosidic bonds) was determined it was found that pullulan partially protected both pullulanase and α -amylase from EDAC modification. Similarly, amylose partially prevented inactivation of both activities (Table 1). The data suggest that hydrolysis of α 1-6 linkages of pullulan and α 1-4 linkages of amylose occurred at a common EDAC-sensitive site.

In order to test if pullulanase and α -amylase activities

Table 1. Protective effect of substrates on modification of *Thermoanaerobium pullulanase* by EDAC

Enzyme (final concn. 0.15 $\mu\text{g/ml}$) was incubated 40 min in buffer containing 25 mM-phenethylamine and 16 mM-EDAC in the presence or in the absence of 0.3 mg of pullulan or amylose/ml. Reaction was quenched by diluting 20-fold with water and residual activity on pullulan and amylose was determined.

	Residual activity (%)	
	Pullulanase (α 1-6-bond hydrolysis)	α -Amylase (α 1-4-bond hydrolysis)
Unmodified enzyme control	100	100
EDAC-modified enzyme	0	0
EDAC-modified enzyme + pullulan	39	30
EDAC-modified enzyme + amylose	35	22

were located at a single active site or separate independent sites, experiments were performed in which both substrates were present simultaneously, and the data were fitted to the following relationships [4].

If there are two independent sites:

$$v = \frac{V^A \cdot [A]}{K_m^A + [A]} + \frac{V^P \cdot [P]}{K_m^P + [P]}$$

If there is a single site:

$$v = \frac{V^A \cdot [A]}{K_m^A \left(1 + \frac{[P]}{K_m^P}\right) + [A]} + \frac{V^P \cdot [P]}{K_m^P \left(1 + \frac{[A]}{K_m^A}\right) + [P]}$$

where A and P represent amylose and pullulan, [A] and [P] are substrate concentrations, K_m^A and K_m^P are apparent Michaelis constants, V^A and V^P are maximal velocities at substrate saturation for A and P respectively and v is the rate of product formation.

Fig. 4 shows a typical result in which [A] was held constant at $0.69 K_m^A$, [P] was varied between $0.1 K_m^P$ and $1.4 K_m^P$ and the observed value of v was compared with v predicted by either model. Observed rates of product formation closely matched those predicted by the single-site model.

The observation that 0.5 mM-cyclomaltoheptaose inhibited hydrolysis of pullulan and amylose by 70% and 40% respectively compared with controls supports the single-site model. Cyclomaltohexaose and cyclomalto-octaose had no effect on either activity.

Hydrolysis pattern

Analysis by h.p.l.c. of the products of pullulan hydrolysis demonstrated that initial cleavage of the polysaccharide chain occurred internally at sites remote from the terminal residues, releasing longer-chain-length oligosaccharides, MT4, MT3 etc. (Fig. 5a), which are subsequently hydrolysed to MT1. Enzyme action on pullulan therefore apparently follows a random endo mechanism.

Fig. 4. Plot of v against $[P]$ for *Thermoanaerobium pullulanase* in the presence of amylose

$[A]$ was held constant at 0.0113 mg/ml and v was determined as $[P]$ was varied in the range 0.0075–0.1 mg/ml. $\circ$, Observed value of v ; ----, line calculated from two-site model; —, line calculated from single-site model, taking K_m^A and K_m^P as 0.016 and 0.073 mg/ml respectively and V_m^A and V_m^P as 106 and 543 nmol/min per ml respectively.

The hydrolysis pattern of short-chain amylose (containing about 22 glucose residues) was studied by simultaneously comparing the rate of reducing-sugar formation with loss of I_2/KI staining capacity of the substrate (Fig. 6). Endo-amylases (e.g. α -amylase) show a rapid decrease in absorbance of the I_2 -amylose complex as compared with exo-amylases (e.g. β -amylase) [15]. The response of the *Thermoanaerobium* enzyme was

similar to that of commercial bacterial α -amylase. H.p.l.c. analysis of intermediates of amylose hydrolysis revealed complex mixtures of oligosaccharides (Glc2–Glc10 plus higher malto-oligosaccharides) that were not adequately resolved by h.p.l.c., indicating that enzyme action on amylose follows a random endo mechanism.

Glc2 and Glc3 were not effective substrates for the enzyme. Glc4 was very slowly hydrolysed, yielding Glc2 as product, but Glc5 was readily hydrolysed to Glc2 and Glc3. Fig. 5(b) shows that Glc4 and Glc2 were the products of enzyme action on Glc6. (Production of Glc2 from Glc4 was insignificant under the experimental conditions.) Glc7 was initially broken down to Glc2 and Glc5 (Fig. 5c), which was subsequently hydrolysed to Glc3 and Glc2. It was concluded that enzyme action on malto-oligosaccharides containing seven or fewer glucose residues occurs at α 1–4-glucosidic linkages, two glucose residues away from a terminal, and releases Glc2 as product.

H.p.l.c. analysis of the intermediates of enzyme action on branched glucans (potato amylopectin, oyster glycogen) revealed malto-oligosaccharide homologues of Glc2 to Glc6. This product profile is consistent with enzymic hydrolysis occurring at both α 1–6 branch points and inter-branch-point α 1–4-glucosidic linkages.

Specificity

A quantitative comparison of the specificity of the enzyme for different substrates was attempted by comparing the ratios of the catalytic centre activity, k_{cat} (number of molecules of reducing sugar product formed/min per catalytic centre) with apparent K_m (mg/ml). Values of k_{cat}/K_m and K_m were respectively: pullulan, 4.0×10^6 and 0.07; MT2, 1.1×10^6 and 0.24; corn amylopectin, 1.4×10^6 and 0.03; potato amylopectin, 5.0×10^6 and 0.01; rabbit liver glycogen, 2.2×10^6 and 0.04; oyster glycogen, 6.0×10^6 and 0.01; Lintner's starch, 1.3×10^6 and 0.05; short-chain amylose, 1.5×10^6 and 0.02; potato amylose, 2.6×10^6 and 0.02.

Fig. 5. Hydrolysis pattern of *Thermoanaerobium pullulanase* activity on pullulan and malto-oligosaccharides

Enzyme was incubated with substrate as described in the text and the products were analysed by h.p.l.c. (a) Enzyme action on pullulan: MT1 ($\square$), MT2 ($\blacksquare$), MT3 ($\circ$), MT4 ($\bullet$) and pullulan ($\triangle$). (b) Enzyme action on Glc6: Glc2 ($\circ$), Glc4 ($\square$) and Glc6 ($\triangle$). (c) Enzyme action on Glc7: Glc2 ($\circ$), Glc3 ($\bullet$), Glc5 ($\blacksquare$) and Glc7 ($\blacktriangle$).

Fig. 6. Comparison of reducing-sugar products of amylose hydrolysis with I_2/KI staining capacity

Short-chain amylose was incubated with *Thermoanaerobium* pullulanase (■), commercial α -amylase (▲) or commercial β -amylase (●) as described in the text. Samples were withdrawn at time intervals and I_2/KI staining capacity and reducing-sugar products were determined.

DISCUSSION

Enzyme action on $\alpha 1-6$ -glucosidic linkages of pullulan and the $\alpha 1-4$ -glucosidic bonds in amylose involves the same active site. The α -amylase from *Thermoactinomyces vulgaris* is able to hydrolyse some of the $\alpha 1-6$ linkages in partial hydrolysates of pullulan as well as $\alpha 1-4$ linkages in pullulan and starch [16] at a single active site [4]. However, the action of the *Thermoactinomyces vulgaris* enzyme on pullulan is primarily against $\alpha 1-4$ linkages, yielding panose as product [16], whereas the *Thermoanaerobium* enzyme only hydrolyses $\alpha 1-6$ bonds in pullulan and yields maltotriose.

Thermoanaerobium pullulanase is apparently active not only on linear $\alpha 1-6$ linkages of pullulan but also on branch-point $\alpha 1-6$ bonds of amylopectin and glycogen. Debranching enzymes may generally be classified as pullulanases if they are active on pullulan and amylopectin but not glycogen, and as isoamylases (EC 3.2.1.68) if they attack glycogen and amylopectin but not pullulan [2]. Comparison of k_{cat}/K_m values for pullulan, amylopectins and glycogens reveals them all to be effective substrates, and the enzyme has properties in common with both isoamylase and pullulanase as well as attacking $\alpha 1-4$ linkages in an α -amylase-like manner.

The highly branched nature of glycogen is considered to impose structural restraints preventing access of pullulanases to branch-point $\alpha 1-6$ linkages [17,18]. The ability of the *Thermoanaerobium* enzyme to attack $\alpha 1-4$ bonds remote from branch points could provide a mechanism by which this structural restraint is relieved, thereby promoting accessibility to the branch points.

Enzyme action on MT2 is slower than on higher MT series oligosaccharides and pullulan (Fig. 5a). The high value of K_m for MT2 compared with pullulan (0.24 compared with 0.07 mg/ml) suggests that the hexa-saccharide binds with lower affinity than does polymeric pullulan. Some polysaccharides are known to have large

binding sites that span several oligomeric sugar residues (e.g. *Aspergillus saitoi* glucoamylase requires co-ordination of seven glucose residues for optimal activity [19]). We tentatively propose that MT2 is too small a substrate to occupy fully and co-ordinate correctly with the binding site.

Similar considerations may explain why hydrolysis of $\alpha 1-4$ -glucosidic linkages of malto-oligomers is effectively restricted to oligosaccharides of chain length five or more residues. Enzyme action on Glc5 is slower than on Glc7 (Fig. 5c), and Glc4 is a poor substrate.

The observation that malto-oligosaccharide hydrolysis involves an exo mechanism rather than the endo mechanism exhibited with amylose is interesting. We speculate that, if the binding site spans several glucose residues, polymeric substrates would fully satisfy binding requirements and would be optimally co-ordinated with the catalytic centre. Smaller oligomeric substrates could not fully occupy the binding site and would co-ordinate in multiple binding modes determined by sub-site affinities [19]. Maltogenic exo hydrolysis of oligomers could readily be explained by assuming that, for a substrate that does not completely fill the binding site, the sub-site affinities favour a binding mode in which two glucose residues overlap the catalytic centre.

REFERENCES

- De Mot, R., Van Oudendijk, E. & Verachtert, H. (1984) *Biotechnol. Lett.* **6**, 581–586
- Fogarty, W. M. & Kelly, C. T. (1979) *Prog. Ind. Microbiol.* **15**, 87–151
- Plant, A. R., Morgan, H. W. & Daniel, R. M. (1986) *Enzyme Microb. Technol.* **8**, 668–672
- Sakano, Y., Hiraiwa, S., Fukushima, J. & Kobayashi, T. (1982) *Agric. Biol. Chem.* **46**, 1121–1129
- Morgan, H. W., Patel, B. K. C. & Daniel, R. M. (1985) *FEMS Microbiol. Lett.* **30**, 121–124
- Plant, A. R., Patel, B. K. C., Morgan, H. W. & Daniel, R. M. (1987) *Syst. Appl. Microbiol.* **9**, 158–162
- Plant, A. R., Clemens, R. M., Daniel, R. M. & Morgan, H. W. (1987) *Appl. Microbiol. Biotechnol.*, in the press
- Abdullah, M. & French, D. (1970) *Arch. Biochem. Biophys.* **137**, 483–493
- Lever, M. (1973) *Biochem. Med.* **7**, 274–281
- Hoare, D. G. & Koshland, D. E. (1967) *J. Biol. Chem.* **242**, 2447–2453
- Carraway, K. C. & Koshland, D. E. (1972) *Methods Enzymol.* **25**, 616–623
- Carraway, K. C. & Koshland, D. E. (1968) *Biochim. Biophys. Acta* **160**, 222–224
- Levy, H. M., Leber, P. D. & Ryan, E. M. (1963) *J. Biol. Chem.* **238**, 3654–3659
- Kochhar, S. & Dua, R. D. (1984) *Biosci. Rep.* **7**, 613–619
- Banks, W., Greenwood, C. T. & Khan, K. M. (1970) *Carbohydr. Res.* **12**, 79–87
- Fukushima, J., Sakano, Y., Iwai, H., Itoh, Y., Tamura, M. & Kobayashi, T. (1982) *Agric. Biol. Chem.* **46**, 1423–1424
- Gunja-Smith, Z., Marshall, J. J., Smith, E. E. & Whelan, W. J. (1970) *FEBS Lett.* **12**, 96–100
- Mercier, C., Frantz, B. M. & Whelan, W. J. (1972) *Eur. J. Biochem.* **26**, 1–9
- Koyama, T., Inokuchi, N., Kikuchi, Y., Shimada, H., Iwama, M., Takahashi, T. & Irie, M. (1984) *Chem. Pharm. Bull.* **32**, 757–761