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The amylopullulanase of *Bacillus* sp. DSM 405

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Abstract The amylopullulanse produced by *Bacillus* sp. DSM 405 was purified to homogeneity. It exhibited dual activity, cleaving the α1-4 bonds in starch, releasing a range of malto-oligosaccharides, and also cleaving the α1-6 bonds in pullulan, releasing maltotriose as the sole end-product. The enzyme was a glycoprotein and had a relative molecular mass of 126 000 and an isoelectric point of 4.3. While the enzyme was optimally active on starch at pH 6.5 and at pH 6.0 on pullulan, activity on both substrates was maximal at 70 °C. Kinetic analyses of the enzyme in a system that contained both starch and pullulan as two competing substrates demonstrated the dual specificity of the enzyme. Chemical modification of the carboxyl groups within the active centre of the protein showed that one active site was responsible for hydrolysis of the α1-4 and α1-6 bonds in starch and pullulan respectively. This is the first comprehensive investigation of an amylopullulanse produced by an aerobic bacterium, showing a single active site responsible for both activities.

Introduction

Amylolytic enzymes degrade starch by hydrolysing either α1-4 and/or α1-6 glucosidic linkages. They can differ greatly in their specificity and properties depending upon their source (Fogarty and Kelly, 1990). In the last decade there have been reports of novel enzymes possessing both starch- and pullulan-hydrolysing activities. The latter is a polysaccharide formed by the linkage of maltotriose units through α1-6 glucosidic bonds. A clear division may be seen in these dual-substrate-hydrolysing enzymes, based on the nature of the bonds cleaved and the end-products formed on hydrolysis of pullulan. Those that hydrolyse α1-4 glucosidic linkages in starch and pullulan and release panose from the latter may be termed panose-producing enzymes (Kuriki et al. 1988). Those degrading α1-4 glucosidic linkages in starch and α1-6 bonds in pullulan, releasing maltotriose, are most frequently called amylopullulanases (Takasaki 1987; Ara et al. 1995a) though other titles have been suggested. The amylopullulanase from the anaerobe *Clostridium thermohydrosulfuricum* appears to possess a single site for both activities (Mathupala et al. 1990) while two active sites are responsible for the dual activities in the aerobic bacteria *Bacillus* sp. KSM-1378 (Ara et al. 1995b, 1996); and *Bacillus circulans* F-2 (Kim and Kim 1995; Sata et al. 1989). A further division of amylopullulanases, based on the number of active sites within the protein, was proposed by Ara et al. (1995b). The pattern of action of the *Bacillus* sp. DSM 405 enzyme suggests that it should be classified as an amylopullulanase. Investigations to determine the number of active sites present in this enzyme are presented in this paper.

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Materials and methods

Microorganism and preparation

Bacillus sp. DSM 405 was obtained from Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany, and was maintained at room temperature on slopes of nutrient agar plus starch (1% w/v) pH 7.0. The microorganism was grown on plates of the same medium at 60 °C for 18 h prior to inoculation into shake-flask culture. The inoculum medium had the following composition (g/l): brain/heart infusion medium 1.0, starch 0.5, glucose 0.2, NH_4NO_3 0.5, KH_2PO_4 0.5, NaCl 0.25, $MgSO_4 \cdot 7H_2O$ 0.25, $FeSO_4 \cdot H_2O$ 0.05, $CaCl_2 \cdot 2H_2O$ 0.05 and 1 ml/l 1 M Na_2SiO_3, pH_i 7.0. The medium for production had the following composition (g/l): corn steep liquor 1.0, maltose 1.0, NH_4NO_3 1.0, KH_2PO_4 0.5, NaCl 0.25, $MgSO_4 \cdot 7H_2O$ 0.25, $FeSO_4 \cdot 7H_2O$ 0.05, $CaCl_2 \cdot 2H_2O$ 0.05 and 1 ml/l 1 M Na_2SiO_3, pH_i 7.0. All media were sterilised by autoclaving at 121 °C for

20 min. *Bacillus* sp. DSM 405 was inoculated from 18-h-old streak plates into 50 ml inoculum medium in 250-ml conical flasks and incubated in shake flask culture at 60 °C for 2 h. Actively growing cells were diluted to give an absorbance reading of 0.3 at 650 nm in a Vitatron Universal photometer. A 1% (v/v) inoculum of this diluted suspension was added to 50 ml production medium in 250-ml conical flasks. The cultures were shaken at 200 rpm in a New Brunswick Orbital incubator (model G25) at 60 °C. Cells were removed from the medium after 18 h incubation, by centrifugation at 16 300 *g* for 10 min at 4 °C in a Sorvall RC2-B centrifuge and enzyme activity in the cell-free supernatant was measured.

Measurement of biomass

In liquid media, biomass was determined in absorbance units at 650 nm in a Universal Vitatron photometer, using water as a blank.

Assay methods

The substrate was prepared in 0.1 M phosphate buffer, pH 6.0 (unless otherwise stated in the text). Usually, 0.5 ml enzyme solution was added to 0.5 ml substrate (1%, w/v) and incubated for 30 min at 40 °C. The reaction was stopped and the reducing sugars determined with dinitrosalicyclic acid according to the method of Bernfeld (1955). An enzyme unit is defined as the amount of enzyme releasing 1 μg glucose equivalents from the substrate/min at 40 °C. All assays were carried out at 40 °C since activity at this temperature was double that at 30 °C.

Determination of protein

Protein was determined at 220 nm in a LKB Biochrom Ultrospec II model 4050 spectrophotometer and related to bovine serum albumin standards.

Enzyme purification

The enzyme was purified through a series of purification techniques including corn starch adsorption, hydrophobic interaction chromatography, ultrafiltration and gel filtration.

Corn starch adsorption

Corn starch (8% w/v) was added to cell-free supernatant followed by industrial methylated spirits (2.5% v/v) at room temperature. The mixture was then filtered through Whatman no. 2 filter-paper to collect all the starch-bound enzyme. The starch cake was incubated for 1 h at 40 °C before being resuspended in 0.02 M phosphate buffer (containing 10 mM $CaCl_2$) at pH 6.0. The enzyme was desorbed from the starch suspension by incubation at 200 rpm in a New Brunswick Orbital incubator for 2 h at 50 °C, followed by centrifugation at 16 300 *g* for 15 min. The desorbed enzyme was dialysed against tap water for 6 h, before concentration by ultrafiltration.

Hydrophobic interaction chromatography

Degassed phenyl-Sepharose CL-4B was used. The resin was poured into a Pharmacia column (3.2 × 6 cm) and washed with the equilibrating buffer (0.05 M BISTRIS propane and 2 M KCl at pH 7.0) until the resin had reached the desired pH. The enzyme sample was pre-filtered through 0.45-mm acrodiscs before being applied to the column. After washing through 500 ml binding buffer, the enzyme was eluted from the column by the elution buffer (0.05 M BISTRIS propane at pH 7.0). The enzyme was dialysed against two changes of 500 ml elution buffer for a total of 4 h to remove any residual salt, and further concentrated by ultrafiltration.

Gel filtration (molecular-sieve chromatography)

A Pharmacia fast protein liquid chromatography (FPLC) Superose 12 prep column (1.6 × 40 cm) connected to a Pharmacia FPLC system was washed with the equilibrating buffer (0.05 M BISTRIS propane at pH 7.0) until the resin had reached the desired pH. The enzyme sample was pre-filtered through 0.22-μm acrodiscs before being applied to the column by means of a valve injection system.

Demonstration of the homogeneity of the purified enzyme preparation by electrophoresis

The homogeneity of the enzyme preparation was demonstrated, using the method of Weber and Osborn (1975), by Phast Gel electrophoresis in a Pharmacia Phast system. Native polyacrylamide gel electrophoresis (PAGE) was also carried out in the Pharmacia Phast system. In both cases proteins were made visible by silver staining. Zymograms were prepared according to the method of Schmidt and John (1979). The enzyme sample was run on a single native PAGE gel and while one-third of the gel was silver-stained the other two-thirds of the gel were used to determine the location of the α-amylase and the pullulanase activities on starch/agar and pullulan-red/agar plates (Yang and Coleman 1987).

Determination of glycoprotein

The enzyme was stained for the presence of a carbohydrate moiety after electrophoresis by the method of McGuckin and McKenzie (1958).

Determination of the isoelectric point

Isoelectric focusing was carried out on Phast Gel IEF3-9 according to the Phast Separation Technique File no. 100. Protein was visualised by silver staining.

Determination of end-products formed

End-products were determined by the methods previously used by Collins et al. 1993.

Kinetics of competitive inhibition with mixed substrates

Various amounts of pullulan were added to three fixed amounts of starch and the enzyme activity at each level was determined. The results were analysed by the kinetic models of Hiromi et al. (1966) to determine the number of active sites present on the protein.

Chemical modification

A modification of the method for carbodiimide-mediated modification of the carboxyl groups in the amylopullulanase of *Thermoanaerobium* Tok6-B1 (Plant et al. 1987) was used to modify the *Bacillus* sp. DSM 405 amylopullulanase, utilising the combined nucleophile and buffer system of semicarbazide and cacodylate used in the modification of bovine pancreatic phospholipase A_2 (Fleer et al. 1981). The enzyme was treated with various concentrations of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) at 20 °C in 0.25 M semicarbazide/cacodylate buffer at pH 4.6. Samples were withdrawn over time and assayed for residual activity, as indicated in the text, and the first- and second-order rate constants calculated. The pK_a of the modified amino acid(s) was determined by carrying out the modification with a set concentra-

tion of EDAC (15 mM) over a range of pH values (4.25–5.5). The pseudo-first-order rate constants of inactivation were determined and pK_a values calculated using non-linear regression analysis.

Results

Physicochemical properties of the amylopullulanase of *Bacillus* sp. DSM 405

Throughout the purification protocol the amylase and pullulanase activities co-eluted, indicating that both activities may be associated with the one protein. The purified enzyme gave a single band on sodium dodecylsulfate (SDS)-PAGE and had specific activities of 11 000 U/mg protein and an approximately 1400-fold increase in purification (Brunswick et al. 1996). The band of purified enzyme obtained on native PAGE was active on starch and pullulan. The enzyme was positively stained by the periodic acid/Schiff reaction, indicating that it was a glycoprotein.

The relative molecular mass (M_r) of the enzyme, determined by gel filtration under non-denaturing conditions and by SDS-PAGE, was 126 000. Thus, this enzyme exhibited dual activities and appeared to be a monomeric protein, closer in size to pullulanases (M_r 50 000–150 000) than to α-amylases (M_r 50 000–60 000). The optimal activities on starch and pullulan were at pH 6.5 and pH 6.0 respectively; both activities had a temperature maximum at 70 °C and the isoelectric point was 4.3.

The half-lives of the enzyme activities at 75 °C were 2.47 min and 2.44 min for the amylase and pullulanase activities respectively; however, the half-lives in the presence of either starch or pullulan were increased up to five-fold (Table 1). As either substrate provided protection for either activity it suggests that, although one protein may be responsible for both activities, there may be one single binding site or active site on the protein that facilitates two different hydrolytic activities.

Pullulan was hydrolysed almost exclusively to maltotriose, indicating that the α1-6 glucosidic linkages in pullulan were hydrolysed. Starch, amylose, amylopectin and glycogen were degraded to a variety of end-products including maltose, maltotriose and maltotetraose. The enzyme was inactive on cyclodextrins. However, the enzyme had a greater affinity for pullulan (K_m 0.41 mg/ml) and amylopectin (K_m 0.64 mg/ml) than for amylose (K_m 1.39 mg/ml). The K_m values were determined at pH 6.5 except for that with pullulan, which was determined at pH 6.0.

Table 1 Determination of the effect of substrates on the half-lives ($t_{1/2}$) of the activities of amylopullulanase of *Bacillus* sp. DSM 405. The enzyme was pre-incubated for 5 min in the absence or presence of substrate at 20 °C in 0.1 M phosphate buffer at pH 6.0. The enzyme was then incubated at 75 °C and samples were removed over time and assayed for residual activities at 40 °C for 30 min, on 1.0% starch (pH 6.5, 0.1 M phosphate buffer) and on 1.0% pullulan (pH 6.0, 0.1 M phosphate buffer). Half-lives were determined from a single-exponential decay equation

Substrate	$t_{1/2}$ (min)	
	α-Amylase activity	Pullulanase activity
No substrate	2.5	2.4
Starch (0.25%, w/v)	11.2	12.0
Pullulan (0.25%, w/v)	12.6	12.5

Kinetics of competitive inhibition with mixed substrates and the number of active sites

Several methods may be used to elucidate the number of active sites present in a protein. In this study kinetic experiments on competitive inhibition with mixed substrates and chemical modification were undertaken to determine the number of active sites present in the amylopullulanase of *Bacillus* sp. DSM 405.

Kinetic experiments on competitive inhibition with mixed substrates were performed at various pullulan concentrations while the starch concentration was held constant at either 0.625 mg/ml, 1.25 mg/ml or 2.5 mg/ml. The initial velocities were determined and were plotted against the total substrate concentration in a S/V versus S plot (where S is the total concentration); only one example is shown for clarity (Fig. 1). The plots will only be linear where one active site is present and, in all three cases, a linear curve was observed. In each of the three cases the value determined for V at a selected substrate concentration fitted the one-site model only (Hiromi et al. 1966), indicating that the amylopullulanase of *Bacillus* sp. DSM 405 may possess a single active site responsible for both activities.

The maximum velocity of an enzyme (V_{max}) obtained in the presence of two substrates equals the sum of the maximum velocities of the substrates when present individually if two active sites are present on the protein, while the maximum velocity obtained in the presence of two substrates should be less than the sum of the

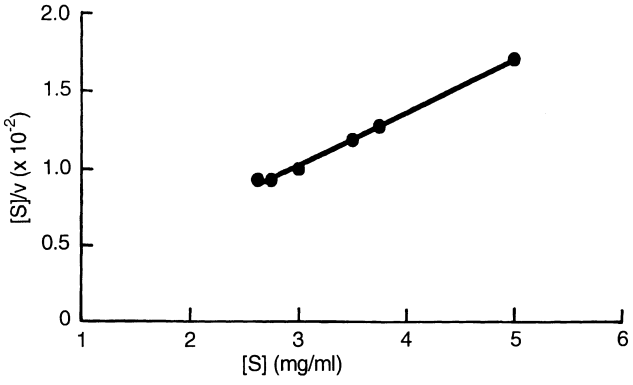

Fig. 1 Kinetics of competitive inhibition with mixed substrates. The amylopullulanase of *Bacillus* sp. DSM 405 was incubated with 2.5 mg/ml starch and increasing levels of pullulan (0.1 M phosphate buffer, pH 6.0) and assayed in the usual manner for 30 min at 40 °C

velocities on the substrates when present individually, if the protein possesses one active site. With this amylopullulanase the V_{max} values obtained in the presence of starch and pullulan alone were 544 units/ml and 788 units/ml respectively, while in the presence of both substrates the V_{max} value obtained was 582 units/ml. These observations strongly indicate the presence of a single active site in the amylopullulanase of *Bacillus* sp. DSM 405.

Chemical modification of the amylopullulanase of *Bacillus* sp. DSM 405

Modification of carboxyl groups in the enzyme was effected by EDAC. Tyrosine groups may also be modified by EDAC; however, this reaction is reversible on addition of hydroxylamine while modification of carboxyl groups is irreversible. Incubation of the partially inactivated enzyme with hydroxylamine did not restore activity on either starch or pullulan, indicating that carboxyl groups and not tyrosine groups were modified by EDAC at pH 4.6. Further evidence that carboxyl groups were involved was obtained by determination of the pK_a of the modified residues. A plot of pseudo-first-order rate constants against pH showed that amino acids with pK_a values of 4.23 and 4.34 were modified, affecting the α-amylase and pullulanase activities respectively. These pK_a values suggest that the amino acid residues modified by EDAC had carboxyl groups, which have a dissociation constant of 4.6 (Lundblad and Noyes 1984).

The effect of the modification on activity was followed over time and at a range of concentrations. A semi-logarithmic plot of residual activity against time yielded straight-line graphs for both α-amylase activity (Fig. 2) and pullulanase activity (Fig. 3), suggesting a first-order reaction. Pseudo-first-order rate constants (k_{obs}) were calculated from the slope of the semi-logarithmic plots of residual activities against reaction time by linear regression analysis. The nature of the reaction mechanism was obtained from the slope of a plot of the negative logarithm of k_{obs} against the negative logarithm of EDAC concentration (Fig. 2 inset, Fig. 3 inset). The value determined for the α-amylase activity was 0.82, suggesting a monomolecular reaction mechanism; however, a lower figure of 0.58 was obtained for the pullulanase activity.

The second-order rate constants for α-amylase inactivation and pullulanase inactivation, determined as 54.0 M^{-1} min^{-1} and 51.8 M^{-1} min^{-1}, were obtained from the slope of the linear plots of pseudo-first-order rate constants against EDAC concentrations (Fig. 4). This indicates that the rate of enzyme inactivation as a function of the concentration of modifier was not dissimilar for the two enzyme activities, suggesting that both activities may be associated with one active site.

Substrates have the ability to block the active site of an enzyme reversibly and thereby protect it against

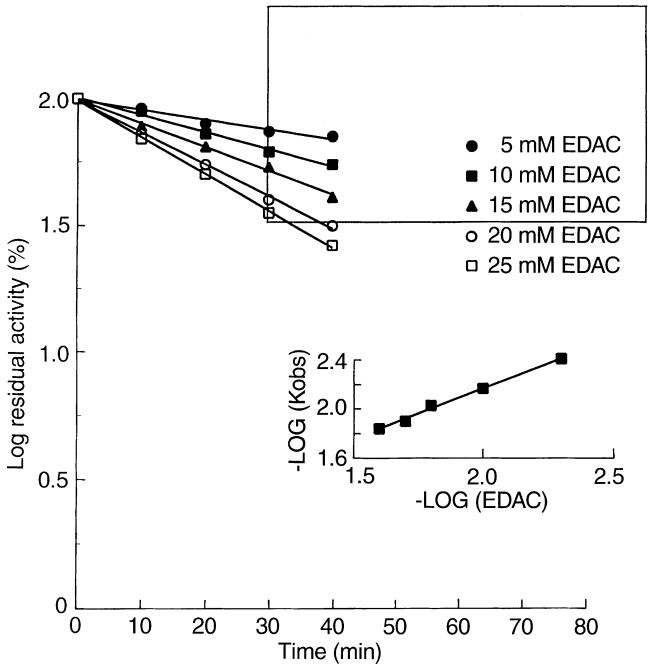

Fig. 2 Effect of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (*EDAC*) on the α-amylase activity of the amylopullulanase of *Bacillus* sp. DSM 405. *Inset*: apparent order of the reaction with respect to modifier concentration. The enzyme was modified with EDAC at the concentrations indicated at pH 4.6 (0.25 M semicarbazide/cacodylate buffer) and 20 °C. The pseudo-first-order constants were determined from these inactivation plots

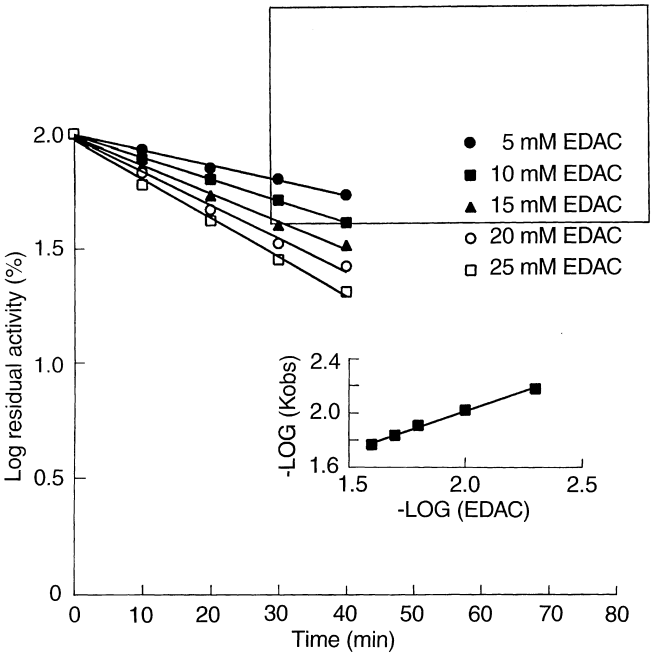

Fig. 3 Effect of EDAC on the pullulanase activity of the amylopullulanase of *Bacillus* sp. DSM 405. *Inset*: apparent order of the reaction with respect to modifier concentration. The enzyme was modified with EDAC at the concentrations indicated at pH 4.6 (0.25 M semicarbazide/cacodylate buffer) and 20 °C. The pseudo-first-order constants were determined from these inactivation plots

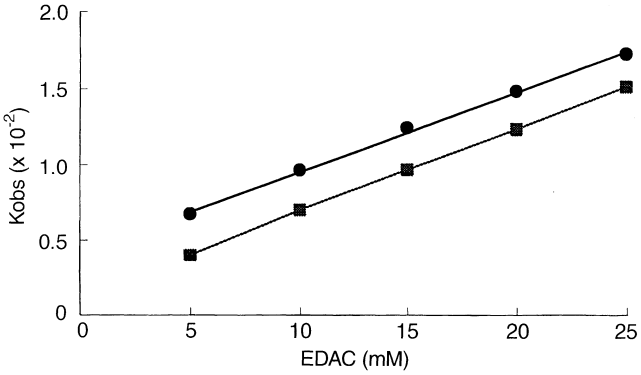

Fig. 4 Effect of EDAC concentration on the observed pseudo-first-order rate constants of inactivation (K_{obs}) of the pullulanase (●) and α-amylase (■) activity of the amylopullulanase of *Bacillus* sp. DSM 405. The enzyme was modified with EDAC at the concentrations indicated, at pH 4.6 (0.1 M semicarbazide/cacodylate buffer), for 40 min at 20 °C. The pseudo-first-order rate constants of inactivation at these concentrations were determined

modification. Protection of the amylopullulanase against EDAC modification, by either substrate, provided further insight into the number of active sites within this enzyme. The presence of either starch or pullulan in the modification mixture protected both enzyme activities from modification to a certain degree, as compared to the unprotected modified mixture, regardless of which substrate was present (Table 2). Each activity was protected to the same extent irrespective of which substrate provided protection, suggesting that both activities are associated with the one site.

Discussion

Novel starch- and pullulan-degrading enzymes are classified on the basis of the end-products produced on the hydrolysis of pullulan. As the enzyme of *Bacillus* sp. DSM 405 degraded pullulan to maltotriose, had the ability to hydrolyse glycogen and was inactive on cyclodextrins, it was classified as an amylopullulanase, as these are traits characteristic of amylopullulanases tested to date (Saha et al. 1990; Kim and Kim 1995). Conversely, panose-producing enzymes have little activity on glycogen and are highly active on cyclodextrins (Kim et al. 1992).

The sheer size of some amylopullulanases, with M_r > 400 000, suggested the presence of a tightly bound enzyme complex of two enzymes, an α-amylase and a pullulanase (Takasaki 1987). However, the unique and distinctive characteristic of amylopullulanases is that one single protein moiety is responsible for both activities. Recent investigations into the active site of the bi-functional proteins – amylopullulanases – led to one further division within starch- and pullulan-hydrolysing enzymes, dividing the amylopullulanases into those that hydrolyse α1-4 and α1-6 linkages at the same active site and those that hydrolyse the two reactions at different active sites (Ara et al. 1995b; Kim and Kim 1995).

Hiromi et al. (1966) developed a method to distinguish whether any enzyme active with two different linkage types had either two active sites, one responsible for each activity, or a single site capable of hydrolysis of both types of linkage. Kinetic equations were provided to determine the number of active sites present in the protein (Mathupala et al. 1990). The amylopullulanase from the aerobic bacterium *Bacillus* sp. DSM 405 appears to have a single active site responsible for both hydrolytic activities, which contrasts sharply with the amylopullulanases from the aerobic bacteria *Bacillus* sp. KSM-1378 and *B. circulans* F-2, which have two active sites, one responsible for each activity (Ara et al. 1995b). Similarly, the amylopullulanse produced by the anaerobic bacterium *Clostridium thermohydrosulfuricum* 39E (Mathupala et al. 1990) also appears to possess a single active site. When an enzyme has two active sites, the model suggests that the apparent maximal velocity, V_{max}, of the mixed substrates must either be equal to the sum of the maximal velocities of the individual substrates, or be greater than the highest individual maximal velocity alone, as observed with the amylopullulanases of *Bacillus* sp. KSM-1378 and *B. circulans* F-2 (Sata et al. 1989; Ara et al. 1995b). Sharply contrasting results were achieved with the *Bacillus* sp. DSM 405 amylopullulanase, whose V_{max} in the presence of mixed substrates was much lower than the V_{max} on pullulan alone. This again was indicative of competition occurring between the two substrates at the active site and further suggested that the *Bacillus* sp. DSM 405 amylopullulanase possessed a single active site for the hydrolysis of both amylolytic activities.

Carbodiimides react with a number of organic functional groups, namely carboxyls, sulfhydryls and tyrosine residues. But the pK_a of carboxylated residues for both activities of the amylopullulanase and the non-reversibility of the modification, strongly suggested that the modified residues were carboxyl groups.

Within one active site, two activities may differ in the level of modifying agent required for inactivation (Pereira and Sivakami 1991), therefore the lower order of reaction obtained for the pullulanase activity of

Table 2 Determination of the protective effect of starch and pullulan on the activities of the amylopullulanse of *Bacillus* sp. DSM 405 in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (*EDAC*). The enzyme was preincubated with 0.25% substrate for 5 min at 20 °C prior to the addition of modifying agents (EDAC, 25 mM in 0.25 M semicarbazide/cacodylate buffer pH 4.6). Residual activity was determined by assaying at 40 °C for 30 min, on 1.0% starch (pH 6.5, 0.1 M phosphate buffer), and 1.0% pullulan (pH 6.0, 0.1 M phosphate buffer)

Treatment	Residual α-amylase activity (%)	Residual pullulanase activity (%)
Unmodified enzyme	100	100
EDAC modified enzyme	32	24
EDAC modified enzyme and starch	75	55
EDAC modified enzyme and pullulan	75	55

Bacillus sp. DSM 405 amylopullulanase may be a result of a faster rate of inactivation, requiring lower amounts of modifier. The second-order rate constants, which describe the rate of inactivation relative to the concentration of the modifier, were shown to be very similar for α-amylase and pullulanase inactivation. This indicated that the two enzyme activities of the *Bacillus* sp. DSM 405 amylopullulanase reacted to the modifier in a similar manner and again led to the suggestion that both activities may be present at the one active site.

Substrates are known to protect enzyme active sites by binding to them reversibly, thereby protecting them against modification (Munch and Tritsch 1990). As protection was afforded to both activities of *Bacillus* sp. DSM 405 amylopullulanase to the same degree by both substrates, this supports the view that both activities must have been present at the same active site, as also observed with the amylopullulanase of *Thermoanaerobium* Tok6-B1 (Plant et al. 1987). In marked contrast, the amylase activity of the *Bacillus* sp. KSM-1378 amylopullulanase was protected from *N*-bromosuccinimide modification of tryptophan residues by amylose only and the pullulanase activity was protected from modification by pullulan alone, indicating the presence of two active sites (Ara et al. 1995b).

The amylopullulanase of *Bacillus* sp. DSM 405 is the first amylopullulanase produced by an aerobic microorganism that has been shown to possess a single active site responsible for the hydrolysis of both the α1-4 glucosidic linkages in starch and the α1-6 glucosidic linkages in pullulan.

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