Biochem. J. (1988) 250, 813–818 (Printed in Great Britain

Purification and some properties of the extracellular α-amylase–pullulanase produced by *Clostridium thermohydrosulfuricum*

Hannes MELASNIEMI
Research Laboratories of the Finnish State Alcohol Company (Alko) Ltd., P.O. Box 350, SF-00101 Helsinki, Finland

The novel α-amylase–pullulanase produced by *Clostridium thermohydrosulfuricum* E 101–69 was purified as two forms (I and II) from culture medium, by using gel filtration in 6 M-guanidine hydrochloride as the final step. Renatured α-amylase–pullulanase I and II had apparent M_r values of 370000±85000 and 330000±85000 respectively, as determined by native polyacrylamide-gradient-gel electrophoresis. Both forms appear to be dimers of two similar subunits, with M_r values of 190000±30000 for enzyme I and 180000±30000 for enzyme II according to SDS/polyacrylamide-gradient-gel electrophoresis. The two forms had similar amino acid compositions, the same *N*-terminal sequence (Glu-Ile-Asp-Thr-Ala-Pro-Ala-Ile) and the same pI of 4.25. Both forms contained sugars having mobilities identical with those of rhamnose, glucose, galactose and mannose. The amount of neutral hexoses relative to protein was 11–12% (w/w) for both forms.

INTRODUCTION

Clostridium thermohydrosulfuricum is an anaerobic thermophile that ferments starch to ethanol (Hollaus & Sleytr, 1972; Wiegel *et al.*, 1979; Parkkinen, 1986). Hyun & Zeikus (1985*a*,*b*,*c*) and Hyun *et al.* (1985) have investigated starch degradation by the strain E39 and reported a pullulanase and a glucoamylase activity from cell extracts of this strain. Melasniemi (1987*a*), on the other hand, reported the production of α-amylase (EC 3.2.1.1), pullulanase (EC 3.2.1.41) and as yet uncharacterized α-glucosidase activities by the strain E 101–69 in both extracellular and cell-associated locations. The extracellular α-amylase and pullulanase activities of the strain E 101–69 were characterized (Melasniemi, 1987*b*): the two activities responded virtually identically to all parameters tested, suggesting that they are functions of a single, novel, thermostable amylase. In the present paper the purification and some properties of this α-amylase–pullulanase enzyme are described.

MATERIALS AND METHODS

Enzyme and protein assays

α-Amylase and pullulanase activities were assayed at pH 5.6 and 85 °C as described by Melasniemi (1987*a*,*b*), by measuring reducing sugar released from amylose or pullulan, respectively, by the Nelson–Somogyi method (Nelson, 1944; Somogyi, 1952). One unit of α-amylase or pullulanase activity is defined as the amount of enzyme releasing 1 nmol of reducing sugar/min in the assay; anhydrous glucose was used as a standard. Protein was measured by the method of Lowry *et al.* (1951), with ovalbumin (grade V, Sigma) as standard.

Enzyme purification

Clostridium thermohydrosulfuricum E 101–69 was grown anaerobically at 68 °C for 40 h in a 22-litre unstirred flask in the complex medium described by Melasniemi (1987*a*), with 2% (w/v) Zulkowsky starch (Merck) as the carbon source, but omitting resazurin and thioglycollic acid. Cells were removed by centrifugation (10000 *g*, 10 min). The cell-free supernatant had α-amylase and pullulanase activities of 640 and 1880 units/ml respectively. The purification was carried out at 4 °C unless otherwise stated, and the preparations were kept at 0 or −20 °C. To the supernatant was added wheat starch (BDH; 15 g/l) and NaN_3 (200 mg/l). The slurry was stirred for 90 h and the starch then allowed to settle. The starch cake was washed with 2 litres of ice-cold distilled water. The enzyme was then eluted from the starch with two 0.5 litre portions of distilled water at 60 °C.

The eluates were made 20 mM with respect to sodium acetate, pH 5.6, and applied to a 2.6 cm × 15 cm DEAE-cellulose (DE 52; Whatman) column equilibrated with the same buffer. The column was washed with the buffer and eluted at 150 ml/h first with 100 mM- and then with 200 mM-NaCl in the same buffer. The fractions pooled were concentrated to 21 ml in an ultrafiltration chamber (Amicon) fitted with an Amicon PM 10 membrane.

The sample was dialysed against 10 mM-potassium phosphate, pH 7.0, and applied to a 2.6 cm × 13 cm hydroxyapatite (Bio-Gel HT, Bio-Rad) column equilibrated with the same buffer. The column was washed with the equilibration buffer and then eluted with a 420 ml linear gradient of 10–400 mM-potassium phosphate, pH 7.0 (10 ml/h). The fractions pooled were concentrated to 1 ml by ultrafiltration.

The concentrated sample was run in 200 μl portions at room temperature and 6 ml/h through a Superose 12 gel-filtration column (Pharmacia) in 50 mM-ammonium acetate, pH 6.5, and the output was passed directly to a Superose 6 column. The enzyme was eluted in two adjacent peaks, giving preparations I and II.

Freeze-dried preparations I and II were dissolved in a denaturing buffer [7.3 M-guanidine hydrochloride (Fluka, no. 50940)/0.1 M-sodium acetate/0.02 M-EDTA/0.5 M-

β-mercaptoethanol, pH 8.1] to about 15 mg of protein/ml. The preparations were incubated for 4 h at 50 °C, after which the pH was adjusted to 5.0 by adding 1 M-HCl. The preparations were then gel-filtered through a Superose 6 column at room temperature, in 200 μl portions with a flow rate of 1 ml/h, with 6 M-guanidine hydrochloride / 100 mM-sodium acetate / 20 mM-β-mercaptoethanol, pH 5.0, as eluent. Fractions were pooled and dialysed extensively against 20 mM-NH_4HCO_3, pH 7.9, to obtain renatured enzyme preparations I and II.

Gradient-gel electrophoresis and isoelectric focusing

The M_r values for purified enzyme I and II were determined by native and SDS/polyacrylamide-gel electrophoresis on PAA 4/30 gradient gels (Pharmacia), according to the manufacturer's instructions, in a Pharmacia GE-2/4 electrophoresis apparatus at 10 °C. The native-gel buffer was 90 mM-Tris/80 mM-boric acid/2.5 mM-EDTA, pH 8.4, and the SDS-gel buffer was 40 mM-Tris/HCl/20 mM-sodium acetate/2 mM-EDTA/0.2% SDS, pH 7.4. Pharmacia High Molecular Weight Calibration Kit proteins (M_r values in parentheses) were used as standards in native gels: thyroglobulin (dimer 669000; monomer 330000), ferritin (apoenzyme 440000; monomer 18500), catalase (tetramer 232000; monomer 60000), lactate dehydrogenase (tetramer 140000; monomer 36000) and albumin (67000); in SDS/gels these were supplemented with rabbit muscle myosin (Sigma; M_r 205000), *Escherichia coli* β-galactosidase and rabbit muscle phosphorylase *a* (Boehringer Mannheim; M_r values 116000 and 94000 respectively). Before staining, the gels were soaked overnight in a solution of 25% (v/v) propan-2-ol containing 10% (v/v) acetic acid to fix the proteins and to remove the SDS, when present. Protein was stained by diffusion overnight in a solution of 0.1% Coomassie Brilliant Blue R 250 (Merck) in 25% methanol/10% acetic acid, and carbohydrate was stained by the periodic acid–Schiff method (Fairbanks *et al.*, 1971).

Isoelectric focusing was done on pI 4.0–6.5 PhastGels (Pharmacia). Samples and Low pI Calibration Kit standards (Pharmacia) were run and stained with $AgNO_3$ on the Pharmacia PhastSystem, according to the manufacturer's instructions.

Analytical gel filtration

A sample of enzyme I (32 μg/100 μl) from the purification stage after gel filtration on Superose 12 and 6 columns was pretreated and run in 6 M-guanidine hydrochloride on a Superose 6 column as described above for preparative samples. The standards (50 μg) used were: thyroglobulin (Pharmacia), β-galactosidase (Boehringer Mannheim), albumin (Pharmacia), lactate dehydrogenase (Boehringer Mannheim) and ribonuclease A (Pharmacia). The distribution coefficient, K_d was calculated as described by Ansari & Mage (1977).

Samples (100 μl) of purified enzyme I (35 μg) and II (45 μg) were run on a Superose 12 column in 50 mM-ammonium acetate, pH 6.5, with a flow rate of 6 ml/h. Thyroglobulin (Pharmacia), apoferritin (Sigma) and catalase (Pharmacia) were used as standards (50 μg), and results were calculated as above.

Amino acid composition and *N*-terminal sequence analysis

Samples (100 μg) of purified enzyme I and II were hydrolysed at 0.5 mg/ml under reducing conditions (Penke *et al.*, 1974) with 3 M-mercaptoethanesulphonic acid (Pierce) for 24 h at 120 °C. The hydrolysates were diluted to contain 2–5 μg of protein per injection (100 μl), and amino acids were separated at 60 °C in a Kontron Liquimat III amino acid analyser equipped with an Interaction AA-511 (Interaction Chemicals, Mountain View, CA, U.S.A.) column. For elution, Buffelute (Pierce) buffers A, B and C (4.5, 3.5 and 7 ml respectively) were used. Amino acids were detected with *o*-phthaldialdehyde (Roth & Hampai, 1973).

For *N*-terminal sequence analysis, samples of purified enzyme I and II (about 350 pmol as subunits) were degraded in a gas-phase sequencer. The proteins were loaded on Polybrene (Sigmal; 3 mg)-treated glass-fibre filters and degraded by the Applied Biosystems O3CPTH program. The released phenylthiohydantoin amino acids were analysed by h.p.l.c. on a 0.46 cm × 25 cm Spherisorb S5 ODS2 (Phase Separations, Queensferry, Clwyd, U.K.) column by using a gradient of acetonitrile in 45 mM-sodium acetate, pH 4.75 (Zimmerman *et al.*, 1977).

Sugars liberated from the enzyme

The sugars attached to the purified enzyme were liberated by hydrolysing a freeze-dried sample of enzyme I containing 0.7 mg of protein with 240 μl of 2 M-HCl for 2.5 h in a boiling-water bath in a Teflon-lined screw-cap tube. The contents were dried under vacuum, and the residue was eluted with 100 μl of acetone. The acetone was evaporated and the eluted sugars were dissolved in 5 μl of distilled water. Samples (1 μl) were then run along with standards for 2 h on a silica gel 60 plate (Merck), impregnated with 0.2 M-NaH_2PO_4, with butan-1-ol/acetone/water (4:5:1, by vol.) as the eluent. The sugars were detected by spraying with an aniline reagent (Hansen, 1975).

RESULTS

Enzyme purification

The purification results are summarized in Table 1. The initial starch-adsorption step separated the α-amylase–pullulanase from most of the extracellular α-glucosidase activity (Melasniemi, 1987*a*), which remained unadsorbed (results not shown). Subsequent chromatography on DEAE-cellulose gave two peaks with α-amylase–pullulanase activity, one containing about 20% of the activity, eluted with 100 mM-NaCl, and a second, containing about 80% of the activity, eluted with 200 mM-NaCl. The enzyme material obtained with the lower salt concentration was in a form which did not enter SDS/12%-polyacrylamide gels made as described by Laemmli (1970), but remained in between the stacking and the separating gels, with a smear of dye in the stacking gel. The enzyme obtained with the higher salt concentration entered the gels, but moved very slowly (results not shown). If samples were not boiled, the activities could be detected from sliced gels after electrophoresis and correlated with the stained bands. The enzyme obtained with 200 mM-NaCl was applied on a column of hydroxyapatite. Although this purification step was not very effective, some bands migrating directly

Table 1. Purification of the extracellular α-amylase–pullulanase (α-Amy–Pul) produced by *Clostridium thermohydrosulfuricum*

	Specific activity (k-units/mg of protein)		Activity (k-units)		Activity ratio	Yield (%)	
	α-Amy	Pul	α-Amy	Pul	Pul/α-Amy	α-Amy	Pul
Culture supernatant	0.11	0.32	13400	39500	2.9	(100)	(100)
Starch adsorption	6.9	19	8100	22700	2.8	60	57
DEAE-cellulose (200 mM-NaCl)	18	51	2500	7000	2.8	19	18
Hydroxyapatite (main peak)	26	72	1000	2800	2.8	7.6	7.2
Superose 12+6							
(first peak = I)	39	101	390	1000	2.6	2.9	2.5
(second peak = II)	36	90	150	380	2.5	1.1	1.0
Superose 6/guanidine							
(I)	20	47	90	220	2.4	0.60	0.45
(II)	40	88	80	180	2.2	0.68	0.55

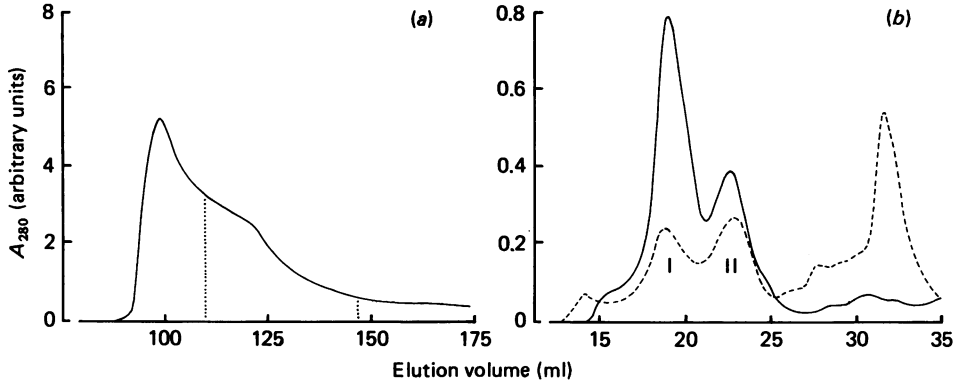

Fig. 1. Chromatography of partially purified α-amylase–pullulanase on hydroxyapatite (*a*) and on combined Superose 12 and 6 columns (*b*)

For details, see the Materials and methods section. (*a*) Pooling of the main peak and the shoulder is indicated by the dotted lines. (*b*) ——, A sample from the hydroxyapatite main peak; ----, a sample from the shoulder. I and II are two adjacent enzyme peaks.

beneath the enzyme in the 12%-acylamide gels were not bound and were removed. From this column the enzyme was eluted at about 100 mM-phosphate in a protein peak having a discernible shoulder (Fig. 1*a*). The enzyme was pooled into two pools according to the degree of purity of the fractions shown in a gel. The pool obtained from the main peak contained more than 70% of the eluted activity and had about twice the specific activity of the pool obtained from the shoulder. Both concentrated pools were gel-filtered on consecutive Superose 12 and 6 columns, to yield in either case two adjacent enzyme peaks, I and II, eluted at the same positions, but having different proportions in the two samples (Fig. 1*b*). Samples from all these four enzyme peaks showed on the 12%-acrylamide gels only two distinct bands, the α-amylase–pulllulanase band in the beginning of the gel and another band with an M_r slightly above 20000. All attempts to separate these two bands in non-denaturing conditions failed. Therefore the enzyme preparations (I and II) obtained from the main hydroxyapatite peak were next gel-filtered in the presence of 6 M-guanidine hydrochloride on a Superose 6 column, which yielded a peak of α-amylase–pullulanase, followed by a smaller peak of the satellite protein. The area under the latter peak (A_{280}) was in both cases about 12% of that of the former. The activities, although very low, could be assayed straight from the effluent. After extensive dialysis, preparation I contained about half of the protein, but only about one-quarter of the activity, compared with the same preparation before the guanidine hydrochloride step, and so its specific activity was lowered by about 50% in this step. Preparation II, on the other hand, also lost about half of its protein but only about half of its activity, and so the specific activity of this preparation remained almost unchanged (Table 1). The dialysed satellite protein shown no α-amylase or pullulanase activity. The final preparation of α-amylase–pullulanase I had a volume of 13 ml, α-amylase and pullulanase activities of 6.9 k-units/ml and 16.3 k-units/ml respectively, and a protein content of 0.35 mg/ml. The final enzyme preparation II had a volume of 4.5 ml, α-amylase and pullulanase activities of 17.8 k-units/ml and 39.7 k-units/ml respectively, and a protein content of 0.45 mg/ml.

The ratio of the pullulanase and α-amylase activities was slightly changed as the enzyme was purified (Table 1). However, at no occasion during the purification did the activities diverge from each other. In addition, the activities could not be separated by ion-exchange chromatography in 8 M-urea, ion-exchange or hydro-

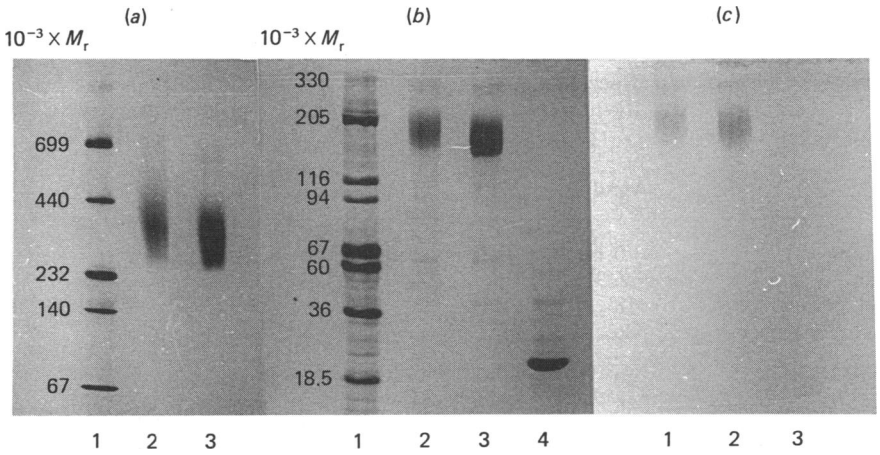

Fig. 2. Electrophoresis of purified α-amylase–pullulanase I and II on 4–30% polyacrylamide-gradient gels

For details, see the Materials and methods section. (*a*) Native gel. Lanes: 1, M_r standards; 2, enzyme I; 3, enzyme II; samples 5 μg of protein. (*b*) SDS gel. Lanes; 1–3, as above; 4, α-amylase–pullulanase satellite protein (see the text); samples 5 μg of protein. (*c*) SDS gel stained for carbohydrate. Lanes: 1, enzyme I; 2, enzyme II; 3, satellite protein; samples 10 μg of protein.

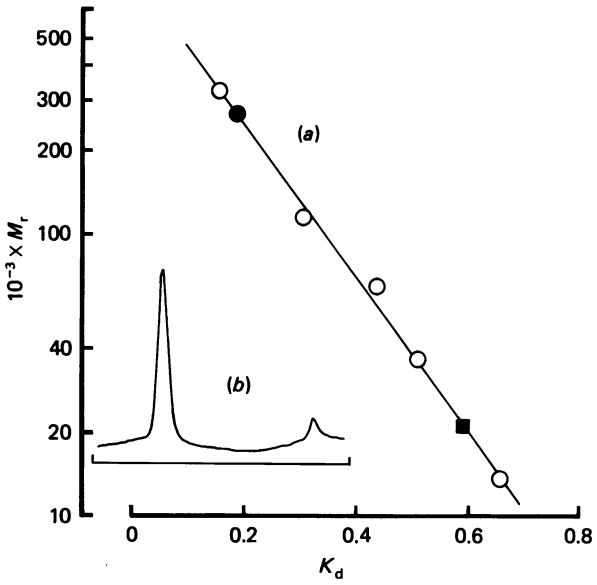

Fig. 3. Gel filtration of reduced α-amylase–pullulanase I and satellite protein in 6 M-guanidine hydrochloride

For details, see the Materials and methods section. (*a*) Plot of M_r (log scale) versus K_d of standard proteins (○), enzyme I (●) and satellite protein (■). (*b*) Elution profile (A_{280}) of enzyme I (left peak) and satellite protein (right peak) (sample, 32 μg of protein).

phobic chromatography in the presence of propan-1-ol, ion-exchange chromatography at 60 °C, or gel filtration at 60 °C in the presence of substrate or under anaerobic conditions.

M_r and pI

Both forms of the purified α-amylase–pullulanase produced strikingly diffuse bands on electrophoresis. The purified enzyme I had an M_r of 370000 ± 85000 and enzyme II an M_r of 330000 ± 85000, as determined by native polyacrylamide-gradient-gel electrophoresis (Fig. 2*a*). Gradient-gel electrophoresis in the presence of SDS (Fig. 2*b*) showed for each form of the enzyme only one kind of subunit, with M_r values of 190000 ± 30000 and 180000 ± 30000 for enzyme I and II respectively. Increasing the amounts of SDS to 2% (w/v), β-mercaptoethanol to 1.5 M and EDTA to 20 mM in the samples and boiling them for 15 min, or performing the electrophoresis in SDS gels containing 8 M-urea, did not cause further dissociation of the subunits (results not shown).

Figs. 2(*a*) and 2(*b*) show the less efficient Coomassie Blue staining of enzyme I as compared with enzyme II on both native and SDS gels. The possibility that the effect was produced by an error in the measurement of protein was excluded by the fact that peptide-bond absorption measurements of the final preparations, and calculations based on the average $\epsilon_{205}^{1\ mg/ml}$ of 31 (Scopes, 1974), gave protein concentrations deviating by less than 1% from those obtained by the Lowry *et al.* (1951) method.

The satellite protein gave an M_r of 24000 ± 2000 on an SDS gradient gel (Fig. 2*b*). The relative peak areas (A_{280}) of the satellite protein and the enzyme, obtained by preparative gel filtration in guanidine hydrochloride, and the M_r values obtained by SDS/polyacrylamide-gradient-gel electrophoresis suggest that the proteins are associated in a 1:1 subunit ratio.

Fig. 3 shows the results from an analytical gel-filtration run of a reduced sample of enzyme I and satellite protein on a Superose 6 column in 6 M-guanidine hydrochloride. The enzyme was eluted as a sharp symmetrical peak, at a position corresponding to an apparent M_r of 275000 ± 20000, whereas the satellite protein showed an M_r of 21000 ± 3000. This method did not resolve enzyme I and II from each other in a sample containing both purified satellite-protein-free forms of the enzyme.

The M_r of the renatured enzyme was also determined approximately by gel filtration on a Sperose 12 column, from which both forms (I and II) were eluted slightly ahead of the apoferritin standard (M_r 443000). This method gave apparent M_r values of about 480000 and 440000 for enzyme I and II respectively. A crude enzyme sample, on the other hand, gave an enzyme peak in

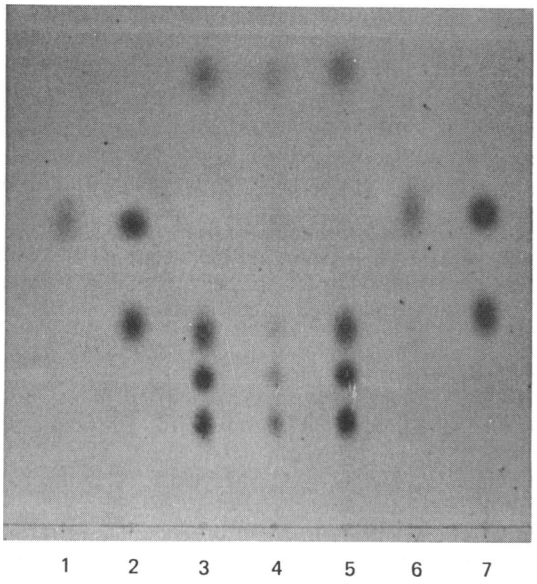

Fig. 4. Thin-layer chromatogram of the sugars liberated from purified α-amylase–pullulanase I

For details, see the Materials and methods section. Lanes (standard sugars from top to bottom): 1, L-fucose (Sigma); 2, D-xylose (Sigma) and L-arabinose (Merck); 3, L-rhamnose (Sigma), D-mannose (Fluka), D-glucose (Merck) and D-galactose (Calbiochem); 4, sugars liberated from enzyme I: 5, L-rhamnose, D-mannose, D-glucose and D-galactose; 6, L-fucose; 7, D-xylose and L-arabinose.

Table 2. Amino acid compositions of α-amylase–pullulanase I and II

Abbreviation: N.D., not determined.

	Amino acid composition (residues/100 residues)	
Amino acid	Enzyme I	Enzyme II
Asx	17.4	15.0
Thr	8.0	8.2
Ser	5.8	6.3
Glx	8.8	9.1
Pro	N.D.	N.D.
Gly	8.9	9.1
Ala	6.1	6.4
Cys	N.D.	N.D.
Val	8.1	7.6
Met	1.5	1.6
Ile	6.8	6.6
Leu	5.8	5.9
Tyr	6.5	7.3
Phe	3.7	4.3
His	0.7	1.4
Lys	8.7	7.8
Trp	N.D.	N.D.
Arg	3.2	3.5

between the peaks of the purified forms, with some enzymically active (aggregated ?) material being eluted in the void volume of the column, indicating an apparent M_r of over 10^6 for this material.

The pI values of the purified enzyme and satellite protein were obtained by isoelectric focusing. Only one band was obtained from each form (I and II) of the enzyme. Both enzyme forms had a pI of 4.25, whereas the pI of the satellite protein was 4.95.

Carbohydrate attached to the enzyme

Both forms of the purified enzyme contained carbohydrate, stainable by the periodic acid–Schiff method after SDS/ polyacrylamide-gradient-gel electrophoresis, whereas the satellite protein did not show carbohydrate staining (Fig. 2*c*). The same Figure shows the less efficient staining of enzyme I as compared with II also by this method. However, no difference to explain this effect was found between the carbohydrate attached to purified enzyme I and II. Neutral hexoses were determined by the anthrone method (Spiro, 1965), with mannose (Alltech Associates) as standard. Enzyme I contained 12% (w/w) and enzyme II 11% neutral hexoses relative to protein. Fig. 4 shows a thin-layer chromatogram of the sugars liberated from purified enzyme I by acid hydrolysis. The neutral hexoses found had mobilities identical with those of rhamnose, mannose, glucose and galactose. In addition, a hardly distinguishable spot was seen in the place of fucose of xylose, not resolved on the plate. Enzyme II gave a similar result (not shown).

Amino acid composition and the *N*-terminal sequence

The amino acid composition of the enzyme (Table 2) showed no unusual features, and was very similar for both forms (I and II), differences being no greater than differences between two independent determinations on the same sample. In agreement with its rather low pI, the protein is fairly rich in Glx. Enzyme I and II had the same *N*-terminal sequence, Glu-Ile-Asp-Thr-Ala-Pro-Ala-Ile.

DISCUSSION

In addition to the two purified forms, the extracellular α-amylase–pullulanase occurred also in a third (aggregated?) form separable by ion-exchange chromatography. The purified enzyme was intimately associated with a satellite protein. The tight association of the proteins and the apparent equimolarity of their constituent subunits suggests that the satellite protein, although not required for the activity of the enzyme, may have some functional relationship to it.

Both forms of the purified enzyme appear to contain covalently linked carbohydrate. The composition of this carbohydrate seems to be similar to that of the S-layer protein on the cell surface of the same species (Sleytr & Thorne, 1976), and under certain cultural conditions the α-amylase–pullulanase also occurs associated with the cell surface (Melasniemi, 1987*a*). Rhamnose, mannose, glucose and galactose, together with arabinose, have also been reported to be associated with purified pullulanase of *Klebsiella pneumoniae* (Shuzheng *et al.*, 1984).

Electrophoresis on polyacrylamide gradient gels in the presence of SDS (Lambin, 1978) and in its absence (Margolis & Wrigley, 1975) seems to give rather reliable subunit and native M_r values even for glycoproteins (see also the leaflet 'Calibration kits for molecular weight determination using electrophoresis'; Pharmacia, 1982). Gel filtration, on the other hand, often yields overestimated M_r values for glycoproteins (Bettelheim, 1977). In random-coil-producing solvents, however, reduced glycopolypeptides have the same hydrodynamic

radius as polypeptides of the same total mass (Leach *et al.*, 1980). The very high M_r values obtained for both forms of the enzyme by gradient-gel electrophoresis in the presence of SDS and in its absence agree well with each other if the native enzyme is assumed to be a dimer. They are also compatible with the apparent M_r values obtained by gel filtration if the latter values are assumed to be somewhat overestimated. The most obvious explanation for the incompatible M_r values obtained by gel filtration in 6 M-guanidine hydrochloride is that the enzyme was not totally denatured by the conditions used, and did not exist as subunits in extended random-coil conformation, but instead as only partly unfolded dimers. Although proteins usually are susceptible to 6 M-guanidine hydrochloride, some proteins are not; this feature appears to be more common with integral membrane proteins (Leach *et al.*, 1980).

Microheterogeneity of the carbohydrate moieties of the enzyme might be one explanation for the marked diffusion of the electrophoretic bands obtained from both purified preparations. However, the average carbohydrate and amino acid compositions of the two forms were very similar and cannot account for the slightly different apparent M_r values and for the differential staining with Coomassie Blue and periodic acid–Schiff reagent. The reason for these differences is not yet known. Differential substitution by, e.g., fatty acyl groups is a possibility that would also explain the occurrence of a proportion of the extracellular enzyme as aggregates of very high M_r, and would be compatible with the cell wall/membrane association of the enzyme observed under certain cultural conditions (Melasniemi, 1987*a*). The extracellular pullulanase of *K. pneumoniae*, initially localized to the outer membrane (Wöhner & Wöber, 1978), is a lipoprotein containing covalently attached fatty acyl groups and is released into the medium in forms of extremely high apparent M_r (Pugsley *et al.*, 1986). In a thermophilic *Clostridium* sp. (Antranikian *et al.*, 1987), on the other hand, increased production of extracellular amylase and pullulanase was paralleled by formation of blebs and vesicles on the surface of the organism, and it was suggested that the activities were localized on this vesicular material.

The possibility that the two forms were produced by proteolytic degradation of a single enzyme is not very likely, for several reasons: (*a*) a low temperature was used during the initial purification steps to keep possible (thermophilic) proteinases inactive; (*b*) the complex medium can be assumed to protect the enzyme by providing a variety of fully denatured substrates for possible proteinases at the high growth temperature used; (*c*) no proteolytic activity could be demonstrated in untreated or sonicated whole-cell cultures grown for 1 or 2 days in the same medium, by incubating these with the general proteolytic substrate Azocoll (Calbiochem) for 4 h at 68 °C, highest absorbance values being obtained from uninoculated medium; (*d*) incubation for 1 h at 37 °C with trypsin, Pronase or proteinase K (0.2 mg/ml) has no effect on the activity of the enzyme in a whole-cell culture; (*e*) the two purified enzyme forms have the same *N*-terminal sequence.

Two other pullulanase–amylase complex enzymes, resembling the α-amylase–pullulanase described here, have been reported, one from a strain of *Bacillus subtilis* (Takasaki, 1987) and the other from a *Thermoanaerobium* sp. (Plant *et al.*, 1987). The latter has, however, not yet been obtained in a homogeneous form, and the homogeneity of the former was demonstrated only under non-denaturing conditions. Thus it is not yet known whether they contain only one or several polypeptide chains.

I thank Dr. Nisse Kalkkinen for sequencing the *N*-terminus of the enzyme, Dr. Kari Suoranta for the amino acid analyses, Dr. John Londesborough for critically reading the manuscript and Ms. Riitta Jussila for technical assistance.

REFERENCES

Ansari, A. A. & Mage, R. G. (1977) J. Chromatogr. **140**, 98–102
Antranikian, G., Herzberg, C., Mayer, F. & Gottschalk, G. (1987) FEMS Microbiol. Lett. **41**, 193–197
Bettelheim, F. A. (1977) in The Glycoconjugates (Horowitz, M. I. & Pigman, W., eds.), vol. 1, pp. 111–128, Academic Press, New York, San Francisco and London
Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) Biochemistry **10**, 2606–2617
Hansen, S. A. (1975) J. Chromatogr. **105**, 388–390
Hollaus, F. & Sleytr, U. (1972) Arch. Mikrobiol. **86**, 129–146
Hyun, H. H. & Zeikus, J. G. (1985*a*) Appl. Environ. Microbiol. **49**, 1168–1173
Hyun, H. H. & Zeikus, J. G. (1985*b*) Appl. Environ. Microbiol. **49**, 1174–1181
Hyun, H. H. & Zeikus, J. G. (1985*c*) J. Bacteriol. **164**, 1146–1152
Hyun, H. H., Shen, G.-J. & Zeikus, J. G. (1985) J. Bacteriol. **164**, 1153–1161
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lambin, P. (1978) Anal. Biochem. **85**, 114–125
Leach, B. S., Collawn, J. F., Jr. & Fish, W. W. (1980) Biochemistry **19**, 5741–5747
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
Margolis, J. & Wrigley, C. W. (1975) J. Chromatogr. **106**, 204–209
Melasniemi, H. (1987*a*) J. Gen. Microbiol. **133**, 883–890
Melasniemi, H. (1987*b*) Biochem. J. **246**, 193–197
Nelson, N. (1944) J. Biol. Chem. **153**, 375–380
Parkkinen, E. (1986) Appl. Microbiol. Biotechnol. **25**, 213–219
Penke, B., Ferenczi, R. & Kovacs, K. (1974) Anal. Biochem. **60**, 45–50
Plant, A. R., Clemens, R. M., Daniel, R. M. & Morgan, H. W. (1987) Appl. Microbiol. Biotechnol. **26**, 427–433
Pugsley, A. P., Chapon, C. & Schwartz, M. (1986) J. Bacteriol. **166**, 1083–1088
Roth, M. & Hampai, A. (1973) J. Chromatogr. **83**, 353–356
Scopes, R. K. (1974) Anal. Biochem. **59**, 277–282
Shuzheng, Z., Suguo, G. & Shoujun, Y. (1984) Ann. N. Y. Acad. Sci. **434**, 282–284
Sleytr, U. B. & Thorne, K. J. I. (1976) J. Bacteriol. **126**, 377–383
Somogyi, M. (1952) J. Biol. Chem. **195**, 19–23
Spiro, R. G. (1965) Methods Enzymol. **8**, 3–26
Takasaki, Y. (1987) Agric. Biol. Chem. **51**, 9–16
Wiegel, J., Ljungdahl, L. G. & Rawson, J. R. (1979) J. Bacteriol. **139**, 800–810
Wöhner, G. & Wöber, G. (1978) Arch. Mikrobiol. **116**, 303–310
Zimmerman, C. L., Appela, E. & Pisano, J. J. (1977) Anal. Biochem. **77**, 569–573

Received 14 July 1987/29 October 1987; accepted 13 November 1987