Cloning and Expression of the *Clostridium thermohydrosulfuricum* α -Amylase-pullulanase Gene in *Escherichia coli*

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An α -amylase-pullulanase gene from *Clostridium thermohydrosulfuricum* DSM 3783 was cloned in *Escherichia coli* on a 7.0 kb *Eco*RI fragment using a λ vector. The gene produced, from an indigenous promoter, active thermostable α -amylase-pullulanase, seemingly mostly a soluble intracellular enzyme in *E. coli*. Gel filtration separated the active enzyme produced into three peaks, each having both α -amylase and pullulanase activities. Immunoblotting after SDS-PAGE revealed more than ten α -amylase-pullulanase specific polypeptides; the biggest of these had an M_r of about 165 000, whereas the smallest enzymically active polypeptide had an M_r of about 100 000. Despite the marked degeneration of its constituent polypeptides, the apparent temperature optimum of the enzyme (80–85 °C) was only some 5 °C lower and the heat stability the same as that of the extracellular α -amylase-pullulanase produced by the native host. Oligonucleotide probes prepared according to the NH₂-terminal amino acid sequences of the enzyme and its satellite polypeptide (a polypeptide associated with the extracellular enzyme of the native host) hybridized to different regions of the 7.0 kb DNA insert.

INTRODUCTION

Starch is composed of glucose units linked through α -1,4 linkages to form chains of varying length. Most of the chains are branched through α -1,6 linkages, the number of which amounts to about 3–4% of the total linkages. Pullulan is also composed of glucose, but in this polymer every third linkage between the constituent glucose units is α -1,6, the rest of the linkages being α -1,4. α -Amylases (EC 3.2.1.1) attack the α -1,4 linkages in starch, whereas pullulanases (EC 3.2.1.41) attack the α -1,6 linkages in pullulan and starch (Fogarty & Kelly, 1979). However, the α -amylases from *Thermoactinomyces vulgaris* (Shimizu *et al.*, 1978) and *Bacillus stearothermophilus* (Suzuki & Imai, 1985) cleave α -1,4 bonds in starch and pullulan. The exo-amylase from *Bacillus* sp. (Suzuki *et al.*, 1987) cleaves α -1,4 linkages in starch and pullulan, and also α -1,6 linkages in starch. Pullulanase/ α -amylase enzymes cleaving α -1,6 linkages in pullulan as well as α -1,4 and α -1,6 linkages in starch have recently been described from *Bacillus subtilis* (Takasaki, 1987), *Thermoanaerobium brockii* (Coleman *et al.*, 1987) and *Thermoanaerobium* sp. (Plant *et al.*, 1987).

Clostridium thermohydrosulfuricum, an obligately anaerobic thermophile, also produces a thermostable and thermoactive enzyme having both α -amylase and pullulanase activities (Melasniemi, 1987*b*, 1988). However, the amount of enzyme activity produced is rather low (Melasniemi, 1987*a*) and in addition the bacterium has complex nutritional requirements (Wiegel *et al.*, 1979). By using recombinant DNA techniques, proteins which are of interest, but obtained with difficulty, can be produced in alternative host organisms. The debranching enzyme of *T. brockii* (Coleman *et al.*, 1987) and a pullulanhydrolase from *B. stearothermophilus* producing not only panose but also maltose and glucose (Kuriki *et al.*, 1988) have recently been cloned. In the present paper the cloning and expression of the *C. thermohydrosulfuricum* α -amylase-pullulanase gene in *Escherichia coli* are reported.

METHODS

Bacterial strains, phages, plasmids and culture conditions. *C. thermohydrosulfuricum* DSM 3783 (E 101-69) was grown as described previously (Melasniemi, 1987a) for 20 h in 2 l of medium containing 2% (w/v) glucose. *EcoRI*-digested dephosphorylated λ gt11 vector DNA and *E. coli* host strains Y1090(r⁻) and Y1089(r⁻) are contained in the Protoclone λ gt11 system (Promega). The host strains are restriction-minus derivatives of strains Y1090 and Y1089, whose use with λ gt11 has been described in detail by Huynh *et al.* (1985).

Plasmids pUC18 and pUC19, differing from each other only in the orientation of their polylinkers, were used for sub-cloning in *E. coli* JM109 (Yanisch-Perron *et al.*, 1985). The polylinkers of the plasmids as well as the unique *EcoRI* site of λ gt11 are located within the β -galactosidase gene adjacent to its promoter, enabling recombinant clones having inserts in these cloning sites to be recognized by the X-gal test.

E. coli JM109 was grown at 37 °C for 16–18 h on Luria-Bertani plates (Maniatis *et al.*, 1982) containing ampicillin (50–100 μ g ml⁻¹) or in the same liquid medium with vigorous shaking.

Isolation of DNA. Chromosomal DNA was isolated from *C. thermohydrosulfuricum* by the phenol method of Amundsen & Neville (1979) modified as follows. Cells (5 g, wet wt) were incubated for 15 min at 37 °C in 25 ml of 50 mM-Tris/HCl pH 8.0 containing 10% (w/v) sucrose, 50 mM-EDTA and lysozyme (5 mg ml⁻¹). Sarkosyl was added to 2% (w/v) and the incubation was continued for 30 min at 65 °C. The isolated DNA was treated with proteinase K and extracted with phenol and chloroform. The solution of the DNA in TE (10 mM-Tris/HCl pH 8.0, 0.1 mM-EDTA) was adjusted to a refractive index of 1.3804 with CsCl and centrifuged at 40000 r.p.m. for 20 h at 20 °C in a Kontron TFT 50.13 rotor with full tubes. The DNA was recovered from the bottom fractions and dialysed against TE. λ DNA was extracted from DNAase- and RNAase-treated polyethylene-glycol-precipitated phage particles (Maniatis *et al.*, 1982). Plasmid DNA was isolated by the method of Birnboim & Doly (1979). For rapid screening of clones the method of Holmes & Quigley (1981) was used.

DNA manipulations. A buffer containing 6 mM-Tris/HCl pH 7.9, 50 mM-NaCl, 6 mM-MgCl₂ and 6 mM- β -mercaptoethanol was used for all restriction enzymes except *EcoRI*, for which 0.1 vol. 1 M-Tris/HCl pH 7.6 was added to the buffer. Analysis of restriction fragments and restriction mapping were done as described by Maniatis *et al.* (1982). For transformation of *E. coli* the method of Hanahan (1985) was used. Restriction enzymes, T4 DNA ligase and DNA standards were from Boehringer Mannheim.

Cloning and sub-cloning. *EcoRI*-digested chromosomal DNA was fractionated by sucrose density gradient centrifugation (Maniatis *et al.*, 1982). Fragments 2–9 kb in size were pooled and ligated to the *EcoRI* site of λ gt11 DNA. The DNA was packed *in vitro* into λ particles by using Packagene (Promega) packaging extracts. Positive plaques on a lawn of Y1090(r⁻) were screened by using a rabbit α -amylase-pullulanase antiserum essentially as described by Huynh *et al.* (1985). The immunocomplexes were visualized by using goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad) together with the chromogenic substrate 3-amino-9-ethylcarbazole (Sarvas & Nurminen, 1985). Screening for enzyme activity was done by overlaying the plates with 5 ml of 0.8% melted agar in 0.2 M-sodium acetate pH 5.2 containing 0.05% soluble starch (Merck 1252) and 2 mM-CaCl₂, incubating for 16 h at 55 °C and staining with iodine vapour. Several positive clones were amplified and concentrated phage stocks were prepared by polyethylene glycol precipitation (Maniatis *et al.*, 1982). Recombinant λ DNA extracted from samples of the stocks was digested with *EcoRI* and the sizes of the inserts were analysed. Y1089(r⁻) was lysogenized with recombinant phage (Huynh *et al.*, 1985) and phage DNA was prepared from induced cells. The insert was excised by *EcoRI*, separated from the λ arms by preparative agarose gel electrophoresis and sub-cloned in the *EcoRI* site on the polylinker of the pUC plasmids.

Oligonucleotide hybridization. Knowing the NH₂-terminal amino acid sequences of the extracellular enzyme from the native host (Melasniemi, 1988) and its satellite polypeptide (H. Melasniemi, unpublished) 27-mer oligonucleotide mixtures complementary to all possible codon combinations for each of the NH₂-terminal sequences were synthesized using a 381A DNA Synthesizer (Applied Biosystems). In the case of the enzyme probe the mixture contained one perfectly matching oligonucleotide species out of a total of 36864 species, in the case of the satellite protein probe one out of 512. The oligonucleotides were labelled with [γ -³²P]ATP using polynucleotide kinase (Maniatis *et al.*, 1982). Hybridization was done at 60 °C directly in a dried 0.8% agarose gel (Miyada & Wallace, 1987) with restriction fragments of the sub-cloned insert resolved on the gel. To eliminate unspecific binding the gels were washed with 0.9 M-NaCl in 90 mM-sodium citrate pH 7.0 for a total of 2 h at room temperature, 5 min at 68 °C and finally for 15 min at 45 °C.

Preparation of cell-free extracts and localization of the enzyme. Transformed JM109 cultures (200 ml) were centrifuged (10000 g, 10 min), and the cells were resuspended in 20 mM-sodium acetate pH 5.6 to 25 ml and passed four times through a French pressure cell (Aminco) at 4 °C using a pressure of about 100 MPa. Soluble and particulate fractions were separated by ultracentrifugation (165000 g, 90 min, at 4 °C) and the particulate pellet was resuspended in the same volume of the buffer. Outer and inner membrane fractions were obtained from the crushed cells as described by Smit *et al.* (1975), except that a protease inhibitor cocktail of phenylmethylsulphonyl fluoride, benzamidine/HCl and *N*-*p*-tosyl-L-lysine chloromethyl ketone (each 1 mM) and pepstatin A (10 μ M) was included in all solutions, and possibly unbroken cells were separated by centrifuging for 20 min at 1200 g.

Periplasmic proteins were released by osmotic shock (Neu & Heppel, 1965) and by a lysozyme/EDTA treatment, in which centrifuged cells were resuspended in 10 mM-Tris/HCl pH 8.0 containing 1 mM-EDTA, 0.5 M-sucrose and lysozyme (5 mg ml⁻¹), incubated for 15 min at 37 °C and diluted with an equal volume of water, after which the spheroplasts were separated by centrifugation (10000 g, 6 min). The periplasmic and intracellular marker enzymes, alkaline phosphatase and glucose-6-phosphate dehydrogenase, respectively, were assayed as described by Malamy & Horecker (1964).

Binding of [¹⁴C]starch. Samples boiled for 2.5 min in the sample buffer were resolved on a 10% (w/v) SDS-PAGE gel (Laemmli, 1970) and blotted onto nitrocellulose essentially as described by Sarvas & Nurminen (1985). The filters were gently shaken, first for 2 h at room temperature in 50 mM-Tris/HCl pH 8.0 containing 150 mM-NaCl and 5% (w/v, dry wt) skim milk for quenching, and then for 16 h on ice with 35 μ Ci (1295 kBq) of [U-¹⁴C]starch (New England Nuclear) in 10 ml of 100 mM-sodium acetate pH 5.6 containing 2 mM-CaCl₂, 0.1 mM-EDTA and 50 mM-NaCl. The filters were washed with ice-cold water, dried and exposed to a Kodak X-Omat XR film.

Enzyme and protein assays. α -Amylase and pullulanase activities were assayed by measuring the amount of reducing sugar released in 15 min from amylose or pullulan as described previously (Melasniemi, 1987a), except that a temperature of 80 °C and a CaCl₂ concentration of 10 mM was used. One unit of activity is defined as the amount of enzyme releasing 1 nmol reducing sugar min⁻¹ in the assay. Protein was measured by the Bio-Rad assay using ovalbumin as a standard.

RESULTS AND DISCUSSION

Cloning of the amylase gene and its promoter region

The *C. thermohydrosulfuricum* genomic library in λ gt11 had a titre of 9×10^5 p.f.u. per μ g of packaged DNA and it contained about 85% recombinant phage particles, approximately 1% of which caused the production of α -amylase-pullulanase. Screening the plates first immunologically and subsequently for enzyme activity revealed roughly equal numbers of positive clones, most of which were detected by both methods. Because the immunological method gave clearer signals and required no replication of the plates, it was used to purify several positive plaques. All purified λ clones had the same 7.0 kb insert, which was subsequently sub-cloned into the *Eco*RI site of pUC18 and pUC19 to give the plasmids pALK351 and pALK353. The restriction map of the cloned fragment is given in Fig. 1. The restriction sites shown are also found in the oppositely oriented polylinkers of the pUC plasmids.

Both λ gt11 and the pUC vectors used are able to express genes inserted in the right reading frame as hybrid proteins from the β -galactosidase promoter upstream of the cloning sites. However, the α -amylase-pullulanase gene is probably transcribed from a *C. thermohydrosulfuricum* promoter on the 7.0 kb insert, because the amount of enzyme activity produced by the recombinant pUC plasmids was not affected by changing the orientation of the insert.

Position and orientation of the amylase gene

The extracellular α -amylase-pullulanase of *C. thermohydrosulfuricum* DSM 3783 occurs in the culture medium in tight, apparently equimolar association with a polypeptide (M_r 24000), the α -amylase-pullulanase satellite polypeptide (Melasniemi, 1988). The synthesized 27-mer oligonucleotide probes for the DNA sequences encoding the NH₂-terminal ends of the enzyme and the satellite protein hybridized specifically to the 1.2 kb *Bam*HI-*Pst*I fragment and the 2.6 kb *Sph*I-*Eco*RI fragment of pALK353, respectively (Fig. 1).

The 0.8 kb *Eco*RI-*Bam*HI fragment from pALK353 (Fig. 1) was deleted by cutting the polylinker and the insert with *Bam*HI and religating to obtain the plasmid pALK355. The insert of this plasmid still has a coding capacity in excess of that required for a polypeptide of the same size as the highest estimate for the subunit of the enzyme of the native host. This deletion had no effect on the amount of activity produced. In contrast, when the 2.6 kb *Sph*I-*Eco*RI fragment was deleted from pALK351 (Fig. 1) by cutting with *Sph*I and religating, no activity was produced. Together with the hybridization data, these results indicate that the promoter region and the 5'-end of the α -amylase-pullulanase structural gene are located on the 1.2 kb *Bam*HI-*Pst*I fragment of pALK353 and that the gene extends onto the 2.6 kb *Sph*I-*Eco*RI fragment.

Fig. 1. Restriction map of the 7.0 kb *C. thermohydrosulfuricum* DNA fragment sub-cloned in the *EcoRI* site of pUC18 and pUC19 plasmids to obtain the plasmids pALK351 and pALK353 respectively. ■, Insert DNA; —, vector DNA; ▲, polylinker of pALK351; △, polylinker of pALK353; arrow, *lacZ'* promoter. The plasmid pALK355 was obtained from pALK353 by deleting the *EcoRI*–*BamHI* fragment adjacent to the polylinker. The regions hybridizing with the α -amylase-pullulanase and the satellite polypeptide probes are indicated by --- and respectively.

Localization of the enzyme in *E. coli*

During growth of *E. coli* JM109 carrying pALK355, pullulanase and α -amylase activities were proportional to the cell mass and reached 2.1 and 0.64 kU ml⁻¹, respectively, after 18 h. This is only slightly more than is produced by the original host (Melasniemi, 1987a). Only about 2% of the total enzyme activity was found in the culture medium. No activity was released by washing the cells with 10 mM-Tris/HCl pH 8.0, and intact washed cells showed less than 1% of the total activity. The bulk of the enzyme activity (98%) was manifested only after complete disintegration of the cells.

About 85% of the activity of the cell-free extracts was found in the soluble fraction and about 15% sedimented on ultracentrifugation. However, when possible association of the sedimented enzyme with outer or inner membrane vesicles was tested by separating the vesicles by sucrose density gradient ultracentrifugation no amylase activity was found in the vesicular fractions; instead the activity was found exclusively in the top fractions of the gradients. The possibility of a periplasmic location was tested. Spheroplasting the cells by lysozyme/EDTA released 80% of the periplasmic marker enzyme alkaline phosphatase, less than 1% of the intracellular marker enzyme glucose-6-phosphate dehydrogenase, and less than 3% of α -amylase-pullulanase. Osmotic shock released more than 75% of the alkaline phosphatase, slightly less than 3% of the glucose-6-phosphate dehydrogenase and about 10% of the α -amylase-pullulanase.

The α -amylase-pullulanase produced by *C. thermohydrosulfuricum* DSM 3783 is bound to the cell surface or is released into the medium, depending on the culture conditions (Melasniemi, 1987a). The present results are most consistent with the idea that the enzyme is produced in *E. coli* as a soluble intracellular protein. Some affinity for the cytoplasmic membrane is suggested by both the sedimentation of a part of the enzyme from homogenates and the slightly higher release of the amylase than of cytoplasmic glucose-6-phosphate dehydrogenase from the cells during the spheroplasting and osmotic shock treatments, which may cause some damage to the cytoplasmic membranes.

Degeneracy of the amylase produced in *E. coli*

Gel filtration of the soluble portion of a cell-free extract of JM109 harbouring pALK355 resolved the α -amylase-pullulanase activity into three peaks (I–III), each showing both α -amylase and pullulanase activities in an approximately 1/3 ratio (Fig. 2). Approximate M_r values of (I) 330 000, (II) 245 000 and (III) 202 000 were obtained from a linear calibration curve established by using thyroglobulin (M_r 669 000), apoferritin (M_r 443 000), catalase (M_r 232 000) and aldolase (M_r 158 000) as standards. The M_r of the enzyme in peak III may, however, be somewhat over-estimated, since deviations from linearity were observed with proteins smaller than aldolase, the smallest protein used for calibration.

Over ten distinct α -amylase-pullulanase specific bands were resolved by immunoblotting from cells carrying pALK355 (Fig. 3). The authenticity of the bands was confirmed by specific binding of [¹⁴C]starch by all of the immunologically detected major bands above M_r 40 000 (results not shown).

Fig. 2. Gel filtration on combined Superose 12 and 6 columns of the α -amylase-pullulanase produced in *E. coli*. A sample (200 μ l) from the soluble fraction of a cell-free extract from JM109 carrying pALK355 was run at room temperature through consecutive Superose 12 and 6 columns (Pharmacia) in 100 mM-sodium acetate pH 5.6 at a rate of 6 ml h⁻¹ and 150 μ l fractions were collected. ■, α -Amylase activity; ●, pullulanase activity; Δ , protein.

Fig. 3. Immunoblotting of the α -amylase-pullulanase produced in *E. coli*. Samples were run on SDS-PAGE (Laemmli, 1970) on a gel containing linear gradients of 4–16% polyacrylamide and 0–18% sucrose in the separating gel. Rabbit muscle myosin, *E. coli* β -galactosidase and Low Molecular Weight Calibration Kit proteins (Pharmacia) were used as standards. Transfer to a nitrocellulose filter and immunostaining was done essentially as described by Sarvas & Nurminen (1985). Lanes [1–3, cell-free extracts from transformed JM109; 4–8, gel filtration fractions (12.5 μ l) from Fig. 2]: 1, unfractionated extract/pUC19 (5 μ l); 2, particulate fraction/pALK355 (2.5 μ l); 3, soluble fraction/pALK355 (2.5 μ l); 4, fr. 179; 5, fr. 184; 6, fr. 191; 7, fr. 196; 8, fr. 199.

The three enzyme peaks obtained by gel filtration (Fig. 2) were composed of different sets of α -amylase-pullulanase polypeptides as judged by the intensities of the bands in the immunoblot in Fig. 3. Peak I contained a major band with M_r about 165 000, which was the highest value observed and probably represents full-length polypeptides. Peak II showed a triplet of bands around M_r 130 000 and peak III a doublet around M_r 100 000. The last value shows that the polypeptide chain can be broken at more than one-third of the way along its length without concomitant loss of activity. These M_r values together with those obtained by gel filtration suggest that the enzyme produced in *E. coli* is dimeric, as is the extracellular enzyme produced by the native host (Melasniemi, 1988). The biggest polypeptide produced by *in vitro* transcription-translation from pALK355 or its 6.2 kb insert alone using the Procaryotic DNA-Directed Translation Kit (Amersham) had an M_r some 20 000 smaller than the M_r of the biggest polypeptide produced *in vivo* (not shown). The same polypeptide patterns were obtained by using either of the templates, confirming that an indigenous promoter was used for transcription *in vitro*.

Immunoblots of samples from the soluble and particulate fractions of the cell-free extracts (Fig. 3) showed that although the quantitative distribution of the α -amylase-pullulanase polypeptides between these fractions was different, most of the immunoreactive polypeptides were present in both. The pattern of degenerate immunoreactive polypeptides seen by SDS-PAGE emerged as a whole at an early stage of culture and did not change during active growth (data not shown). This suggests that a prime cause of the degeneracy is either incomplete translation due to internal initiations or premature terminations, or rapid cleavage of newly synthesized polypeptides, or both. However, as culture was continued to the stationary phase and up to 4 d, the bands at about M_r 43 000, 68 000 and 97 000 became stronger whereas the one at about 57 000, together with all minor bands smaller than 94 000, became weaker. This suggests that a slower partial proteolysis of the various amylase molecules is superimposed on the process(es) causing degeneracy of the newly synthesized polypeptides.

The M_r values observed by SDS-PAGE for the subunits of two purified forms of the α -amylase-pullulanase from the native host (Melasniemi, 1988) were higher than the highest M_r observed in Fig. 3. However, the M_r of the polypeptides produced by the native species may be considerably lower than the M_r values observed, since the enzyme showed several anomalous features and contains secondary modifications including, at least, carbohydrate. A carbohydrate-containing monomeric pullulanase (M_r 136 500) capable of hydrolysing α -1,4 glycosidic linkages has recently been purified by Saha *et al.* (1988) from *C. thermohydrosulfuricum* Z 21-109 by a procedure involving a lipase treatment.

Thermal characteristics of the enzyme

The effect of temperature on the activity of the enzyme produced in *E. coli* was assayed by using a cell-free extract from JM109 carrying pALK355. For the 15 min reaction times used in the presence of 10 mM- Ca^{2+} , the optimum of the α -amylase activity was at about 85 °C and the optimum of the pullulanase activity some 5 °C lower at about 80 °C. Raising the Ca^{2+} concentration did not shift the optima to higher temperatures, but if Ca^{2+} was omitted the optima were shifted some 10 °C lower and at the same time both activities were reduced to about half of that in the presence of Ca^{2+} .

A greater activation by Mn^{2+} than by Ca^{2+} has been reported for the α -amylase and pullulanase activities of *C. thermohydrosulfuricum* DSM 567 (Antranikian *et al.*, 1987). Maximal Mn^{2+} activation of the *E. coli* enzyme at 80 °C was observed at 2 mM- MnCl_2 , and was about 30% for the pullulanase activity and less for the α -amylase activity, whereas activation by Ca^{2+} at the same temperature was about 290% for the pullulanase and about 210% for the α -amylase activity. Over 90% of the maximal activation was observed with only 0.25 mM- Ca^{2+} .

Thermal stability of the enzyme in the absence of substrate is presented in Table 1. In the absence of Ca^{2+} the enzyme was stable at 65 °C, but was rapidly inactivated at 85 °C. In the presence of 10 mM- Ca^{2+} , however, more than 60% of the activity was still left after incubating the enzyme for 2 h at 85 °C. No heat activation similar to that reported for the amylase activities of strain DSM 567 (Antranikian *et al.*, 1987) was evident in experiments with the solubilized *E.*

Table 1. Heat stability of the α -amylase-pullulanase produced in *E. coli*

The soluble fraction of a cell-free extract from JM109 carrying pALK355 was diluted with 100 mM-sodium acetate pH 5.6 to 2.1 kU pullulanase activity, 0.64 kU α -amylase activity and 0.8 mg protein ml⁻¹. Replicate samples were incubated at 55–95 °C in the presence or absence of 10 mM-CaCl₂ for 30 and 120 min. The activity left was assayed as described in Methods using 50 μ l enzyme samples and pullulan as a substrate.

Temp. (°C)	Residual activity (%)			
	30 min		120 min	
	No Ca ²⁺	10 mM-Ca ²⁺	No Ca ²⁺	10 mM-Ca ²⁺
55	93	91	92	90
60	93	90	94	91
65	96	89	96	91
70	94	89	93	91
75	79	87	65	87
80	46	84	15	72
85	8.5	75	0.9	64
90	1.3	58	0.0	22
95	0.5	13	0.1	1.0

coli enzyme, released by effective crushing of the cells. However, increased apparent activities were observed after heat treatment of enzyme entrapped (along with glucose-6-phosphate dehydrogenase) in the membranous matrix of incompletely lysed cells. Such samples showed almost 40% activation after incubation for 2 h at 70 °C in the absence of Ca²⁺ (which stabilizes lipid bilayers) and 25% activation in its presence, perhaps because of slow disintegration of the matrix at elevated temperatures. Some kind of a matrix effect might also explain the activation observed by Antranikian *et al.* (1987) if their enzyme, although extracellular, was not truly soluble.

Considering the marked degeneracy of the enzyme produced in *E. coli*, its thermal characteristics were surprisingly little affected as compared with the enzyme produced by the native host (Melasniemi, 1987*b*): the apparent temperature optimum was only some 5 °C lower and the stability the same. Since an intracellular protein in *E. coli* can fairly safely be assumed to be non-glycosylated, the equal stabilities of the enzyme produced in either host seem to imply that the carbohydrate moiety of the *C. thermohydrosulfuricum* enzyme has no marked role in the thermostability of the enzyme.

The elucidation of the DNA sequence of the α -amylase-pullulanase gene will bring more insight into this enzyme, e.g. reveal possible homology with known α -amylases or pullulanases and show whether the enzyme and the satellite polypeptide are products of only one gene or of two co-regulated genes. The equimolarity and the intimate association of the enzyme and satellite polypeptides produced by *C. thermohydrosulfuricum* favour the idea that these two polypeptides arise in this organism as a consequence of a specific proteolytic cleavage. Despite the degeneracy of the polypeptides observed in *E. coli*, the cloned enzyme retained both its activities as well as its thermal characteristics. This invites further research to produce this novel enzyme by genetically engineered host systems.

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