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Isolation and Characterization of a Heat-Stable Pullulanase from the Hyperthermophilic Archaeon *Pyrococcus woesei* after Cloning and Expression of Its Gene in *Escherichia coli*†

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The gene encoding an extremely heat-stable pullulanase from the hyperthermophilic archaeon *Pyrococcus woesei* was cloned and expressed in *Escherichia coli*. Purification of the enzyme to homogeneity was achieved after heat treatment of the recombinant *E. coli* cells, affinity chromatography on a maltotriose-coupled Sepharose 6B column, and anion-exchange chromatography on Mono Q. The pullulanase, which was purified 90-fold with a final yield of 15%, is composed of a single polypeptide chain with a molecular mass of 90 kDa. The enzyme is optimally active at 100°C and pH 6.0 and shows 40% activity at 120°C. Enzyme activation up to 370% is achieved in the presence of calcium ions and reducing agents such as β-mercaptoethanol and dithiothreitol, whereas *N*-bromosuccinimide and α-cyclodextrin are inhibitory. The high rigidity of the heat-stable enzyme is demonstrated by fluorescence spectroscopic studies in the presence of denaturing agents such as sodium dodecyl sulfate. At temperatures above 80°C, the enzyme seems to switch from the compact to the unfolded form, which is accompanied by an apparent shift in the molecular mass from 45 to 90 kDa.

In the last decade a number of thermophilic microorganisms were found to produce extracellular enzymes that are capable of degrading biopolymers such as starch, hemicellulose, and proteins. The majority of these organisms (extreme thermophiles) grow optimally between 60 and 75°C (2). Very little, however, is known about the physiology and enzymology of microorganisms which grow optimally between 85 and 105°C (hyperthermophiles). All hyperthermophilic microorganisms investigated so far grow to low cell densities and produce low levels of extracellular enzymes. Optimization of growth of *Pyrococcus woesei* and *P. furiosus* has been achieved after cultivation in dialysis membrane fermentors, continuous culture, and air-lift fermentors (47, 48). These hyperthermophilic microorganisms grow on amylacious compounds and form α-amylase, pullulanase, and α-glucosidase (7, 8, 12, 28). The concerted action of these enzymes causes the conversion of polymeric substrates such as maltodextrin and amylose into small sugars such as glucose, maltose, and maltotriose. These sugars are easily transported into the cells and metabolized anaerobically by a fermentative pathway (50, 51). The production of α-amylase and pullulanase seems to be widely distributed among thermophilic, extreme thermophilic, and hyperthermophilic microorganisms (2, 7, 26, 56). All thermoactive pullulanases investigated so far attack both α-1,4- and α-1,6-glycosidic linkages in branched substrates, and they have been named pullulanase type II, or amylopullulanase (39, 49, 55). The pullulanases which hydrolyze preferentially α-1,6-linkages (type I pullulanases) have only been detected in the mesophilic bacteria *Klebsiella pneumoniae*, *Bacillus acidopullulyticus*, and *Bacillus flavocaldarius* (4, 52, 57). In addition to a few extracellular enzymes (e.g., α-amylase and serine protease [14, 27]), a small number of intracellular enzymes have also been investigated in more detail. These include glutamate dehydrogenase, DNA polymerase, α-amylase, β-glucosidase, glyceraldehyde-3-phosphate dehydrogenase, aldehyde-ferredoxin-oxidoreductase, and hydrogenase (9, 11, 23, 32, 36, 41, 59). So far, only five genes encoding thermostable enzymes from hyperthermophiles have been successfully cloned and expressed in mesophilic hosts. These enzymes are glyceraldehyde-3-phosphate dehydrogenase, glutamate dehydrogenase, glutamine synthetase, α-amylase, and DNA polymerase (15, 31, 36, 58, 60). In this communication we present data on the remarkable properties of a heat-stable pullulanase from *P. woesei*. This is the first archaeal pullulanase which has been cloned and expressed in a mesophilic host.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. woesei* (DSM 3773) was obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Federal Republic of Germany [FRG], and was cultivated in a nonturbid, sulfur-free medium of the following composition (in grams per liter): NaCl, 30; KH_2PO_4, 1.4; $(NH_4)_2SO_4$, 1.3; $MgSO_4 \cdot 7H_2O$, 0.25; $CaCl_2$, 0.052; $FeSO_4 \cdot 7H_2O$, 0.038; $Na_2SeO_3 \cdot 5H_2O$, 0.0013; resazurin, 0.001; tryptone, 1.0; yeast extract, 1.0; starch, 1.0; cysteine, 0.5; trace element solution of Belay et al. (3), 10 ml. The pH of the medium was adjusted to 7.0 with 10 M KOH. Cultivation was performed under anaerobic conditions at 90°C, and the inoculum size was 10%. If cells were precultured in Hungate tubes or vials, 1.0 g of $NaHCO_3$ was added to the medium. Fermentation was performed in 15-liter fermentors (Bioengineering, Wald, Switzerland) under continuous gassing with N_2-CO_2 (95%:5%).

Escherichia coli PL2118 expressing the recombinant pullulanase from *P. woesei* was grown aerobically at 37°C in 1-liter flasks containing 0.5 liter of the following medium (in grams per liter): tryptone, 10; yeast extract, 5; NaCl, 10; soluble starch, 10; chloramphenicol, 0.006.

DNA techniques and recombination. *P. woesei* chromosomal DNA was isolated according to Pitcher et al. (43). *P. woesei* DNA, 100 μg, was partially digested with 20 U of *Sau*3A for 10 min at 37°C. The digestion was terminated by phenol-chloroform extraction, and the DNA was ethanol precipitated. The positive selection vector pSJ933 was used for ligation with the chromosomal DNA. Ligation was performed with chromosomal DNA/pSJ933 (digested with *Bam*HI) at a ratio of 1:3, using 4 μg of DNA per 10 μl. Two units of T4 ligase was added and the mixture was incubated at room temperature (25°C) for 4 h. *E. coli* SJ6 (13) was transformed with the ligated DNA and plated on Luria broth plus 2% agar containing 6 μg of chloramphenicol per ml, and the plates were incubated at 37°C. After 16 h of incubation, approximately 14,000 chloramphenicol-resistant colonies were observed on the plates. These colonies were replica

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plated onto a new set of Luria broth plates containing 2% agar, 6 μg of chloramphenicol per ml, and 0.1% dyed pullulan and grown overnight. The plates were then incubated at 60°C for 4 h; a halo appeared around two of the colonies, the result of degradation of the dyed pullulan. Strain SJ6, which lacks the plasmid, showed no pullulanase activity. The corresponding colonies on the first set of Luria broth plates were isolated and analyzed for plasmid content. One of the colonies, PL2118, was grown in 10 ml of Luria broth, and the plasmid was isolated by the method described by Kieser et al. (24). Plasmid pPL2118 was analyzed by restriction mapping and showed an insert of *Pyrococcus* DNA of approximately 4.5 kb. Southern hybridization confirmed that the *P. woesei* DNA from pPL2118 was cloned as a nondeleted continuous fragment from the *P. woesei* chromosome.

Enzyme assay. Pullulanase activity was routinely determined by measuring the amount of reducing sugars liberated when the enzyme was incubated in 50 mM sodium acetate buffer (pH 5.5) at 90°C with 0.5% pullulan (5). One unit of pullulanase catalyzes the formation of 1 μmol of reducing sugars per min under the defined conditions; maltose was used as a standard. Protein was determined according to Lowry et al. (35).

Purification of the recombinant pullulanase. All purification steps were performed at room temperature. *E. coli* cells, 16 g, expressing *P. woesei* pullulanase were washed in 50 mM potassium phosphate buffer (pH 7) and then suspended in 45 ml of the same buffer. Cells were disrupted by sonication, and cell debris was removed by centrifugation for 20 min at 30,000 × *g*. The supernatant was boiled for 20 min, and the denatured host proteins were pelleted by centrifugation (15 min, 30,000 × *g*). The pullulanase remained in the clear supernatant.

Affinity chromatography. The supernatant (about 250 mg of protein) containing pullulanase activity was applied to a maltotriose-Sepharose column (4 by 5 cm). Maltotriose was coupled to epoxy-activated Sepharose as described by Pharmacia (Pharmacia Biotech Europe, Brussels, Belgium). The column was equilibrated with 50 mM sodium acetate buffer (pH 5.5). The pullulanase activity was eluted from the column at a flow rate of 0.3 ml/min with a linear gradient of 75 to 250 mM NaCl in 180 ml of running buffer. Fractions (2 ml) containing high pullulanase activity were pooled and dialyzed against 50 mM Tris HCl buffer (pH 8.5). If necessary, the pool was concentrated by ultrafiltration, using PM10 membranes (Amicon, Beverly, Mass.).

Anion-exchange chromatography. The protein solution (0.2 mg of protein per run) was applied to a Mono Q HR 5/5 column equilibrated with 50 mM Tris HCl (pH 8.5). The pullulanase activity was eluted with an NaCl gradient. Fractions with high activity were pooled, concentrated by ultrafiltration, and finally dialyzed against 50 mM sodium acetate buffer (pH 5.5).

Gel electrophoresis methods. Native gel electrophoresis was carried out according to Jovin et al. (20) with 1-mm-thick slab gels of 7.5% (wt/vol) polyacrylamide in a mini-gel chamber (Biometra, Göttingen, Germany) at 20 mA for 2 h at 4°C. Native polyacrylamide gels containing a gradient of 5 to 27% polyacrylamide were prepared as described by Andersson et al. (1). Gels were run at 300 V for 24 h at 4°C. High-molecular-weight marker proteins (Pharmacia Biotech) were used as standards. In order to examine the subunit composition of the pullulanase, protein samples were also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed in 11.5% (wt/vol) polyacrylamide gels with 0.1% SDS (33). Low-molecular-weight marker proteins (Pharmacia Biotech) were used as the standard. After native and SDS-PAGE, proteins were silver stained as described by Blum et al. (6). Protein bands with pullulytic activity were detected after native PAGE by incubating the gels in sodium acetate buffer (pH 5.5) containing 1% pullulan at 4°C for 1 h. Zymogram staining for pullulytic activity was performed according to Gabriel and Wang (16).

Determination of molecular mass. In addition to SDS-PAGE and linear gradient gel electrophoresis, the pullulanase was subjected to gel filtration chromatography on a Superdex 200 prep-grade column (1.6 by 60 cm; Pharmacia Biotech). The buffer used for gel filtration was 20 mM potassium phosphate buffer (pH 7.0) containing 100 mM NaCl. Furthermore, matrix-assisted laser desorption mass spectrometry was carried out according to Karas and Hillenkamp and Smith et al. (21, 53).

Influence of pH and temperature. For studies on the influence of pH and temperature, experiments were carried out with purified enzyme (0.2 U/ml). The dependence of the pullulanase activity on pH was determined in 50 mM MES (morpholineethanesulfonic acid)-BisTrisPropane buffer (pH 4 to 6.5) and 50 mM BisTrisPropane-MES buffer (pH 7 to 10). The pH of the buffers was adjusted at room temperature. To determine the influence of temperature on pullulanase activity, samples were incubated in screw-cap tubes at temperatures of 40 to 120°C for 10 min. For temperatures above 90°C, an oil bath was used. For determination of the thermostability of the purified enzyme, incubation was performed in screw-cap tubes at 100, 105, and 110°C. At appropriate time intervals, samples were withdrawn and tested for pullulanase activity at 90°C as described above.

Fluorescence spectroscopy. Fluorescence spectroscopy was carried out with purified pullulanase (20 μg/ml; 0.5 U/ml) in a spectrofluorophotometer RF-540 (Shimadzu Corp., Kyoto, Japan) at different temperatures. The excitation wavelength was 280 nm. The fluorescence emission of the enzyme was monitored at 300 to 500 nm.

Analysis of hydrolysis products. Hydrolysis products arising after the action of pullulanase on various linear and branched polysaccharides were analyzed by high-performance liquid chromatography (HPLC) with an Aminex-HPX-42 A column (300 by 7.8 mm; Bio-Rad, Hercules, Calif.). Distilled water was used as the mobile phase at a flow rate of 0.3 ml/min. The purified pullulanase (1.2 U/ml) was incubated at 90°C with 1% (wt/vol) each starch, pullulan, glycogen, amylopectin, amylose, and dextran and 0.5% (wt/vol) panose.

Samples were withdrawn at different time intervals and placed on ice until they were analyzed. Further information on the type of glycosidic linkages in various oligosaccharides was obtained by incubation with α-glucosidase from yeast. This enzyme is capable of hydrolyzing α-1,4- but not α-1,6-linkages in short-chain oligosaccharides.

Effect of metal ions and other reagents. The effect of various substances on pullulanase activity was examined after incubation of the purified enzyme (0.3 U/ml) with metal ions and other reagents in various concentrations at 90°C for 10 min. Aliquots were withdrawn, cooled on ice, and tested for pullulanase activity as described above.

Chemicals. Soluble starch was obtained from Fluka (Buchs, Switzerland). Pullulan, maltose, and panose were obtained from Sigma (St. Louis, Mo.); glycogen from oysters and the oligosaccharide mixture were from ICN (ICN Biomedicals Inc., Costa Mesa, Calif.). Chemicals for electrophoresis were from Serva (Heidelberg, FRG), and α-glucosidase was from Boehringer (Mannheim, FRG). Other chemicals were purchased from Merck (Darmstadt, FRG).

RESULTS

Purification of the cloned pullulanase. The specific activity of the pullulanase of *P. woesei* expressed in *E. coli* was 0.15 U/mg. Denaturation of most of the host proteins was achieved by boiling the cell extract for 20 min. After heat treatment, the sample containing pullulanase activity (250 mg of protein per run) was subjected to affinity chromatography on a maltotriose-coupled, epoxy-activated Sepharose 6B column. The pullulanase was eluted from the affinity column at 200 mM NaCl, and the fractions containing high enzyme activity were pooled (Fig. 1a). After this step, the majority of the contaminating proteins were separated, and the specific activity of the pullulanase was 8.6 U/mg; the enzyme was purified 57-fold. The final purification was achieved by anion-exchange chromatography on a Mono Q column. The enzyme was eluted from the Mono Q column with an NaCl gradient of 300 mM (Fig. 1b). The pullulanase was purified 90-fold, with a specific activity of 13.5 U/mg and a final yield of 15% (Table 1). The pure enzyme was stored at 4°C for at least 4 months without any loss of activity.

Physicochemical properties of the purified pullulanase. Proteins from various purification steps were separated in a native polyacrylamide gel (Fig. 2a). After anion-exchange chromatography on Mono Q, a single protein band was observed (Fig. 2a, lane 4). As shown in Fig. 2b (lane 2), this protein band showed pullulytic activity. Samples from all purification steps were also subjected to SDS-PAGE (Fig. 2c). The sample from the Mono Q pool revealed a protein with an apparent molecular mass of 90 kDa (Fig. 2c, lane 4). Furthermore, the influence of different incubation temperatures on the migration of the protein in an SDS-polyacrylamide gel was investigated (Fig. 2d). After incubation of the homogeneous enzyme with 1% SDS and 1.5% β-mercaptoethanol at temperatures of 4 to 60°C for 10 min (Fig. 2d, lanes 1 and 2), a single protein band with a molecular mass of 45 kDa was observed. Incubation at temperatures above 60°C caused the appearance of a second protein band with a molecular mass of 90 kDa (Fig. 2d, lane 3). Complete denaturation of pullulanase was apparently achieved after boiling for 10 min (Fig. 2d, lane 4).

Further investigations were performed by using matrix-assisted laser desorption mass spectrometry. Since the desorption process does not allow the formation of noncovalent bonds, proteins are not aggregated under these conditions. With this method, the native enzyme was estimated to be 89 kDa. This result is in agreement with the results obtained with SDS-PAGE and linear gradient polyacrylamide gel electrophoresis (5 to 27% [wt/vol] polyacrylamide). Accordingly, the

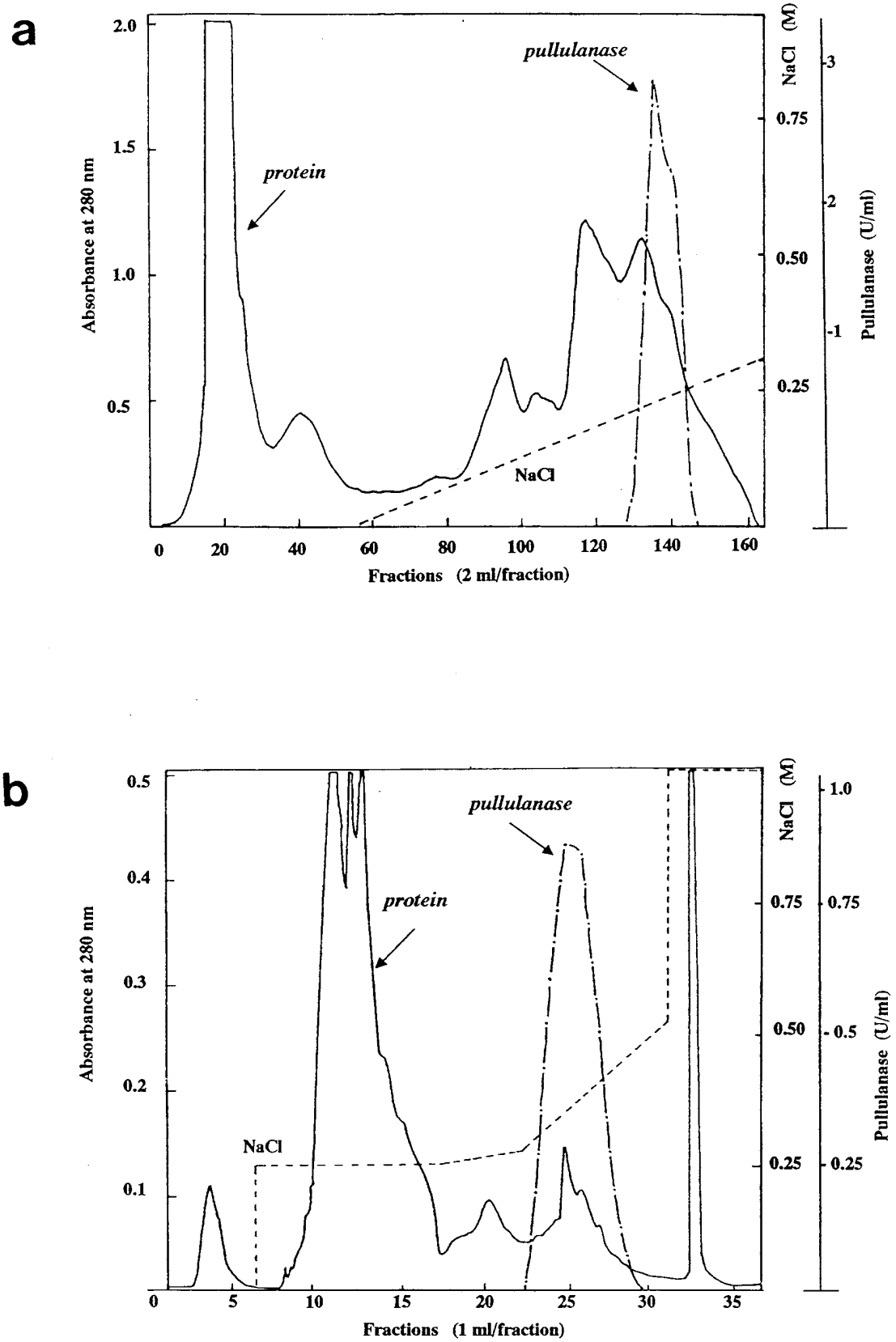

FIG. 1. (a) Affinity chromatography using a maltotriose-coupled Sepharose 6B column. After heat treatment of the recombinant *E. coli* crude extract, 250 mg of protein (50 mM sodium acetate buffer, pH 5.5) was applied to the column (5 by 4 cm), which was run at a flow rate of 0.3 ml/min. The pullulanase was eluted with a linear NaCl gradient. (b) Anion-exchange chromatography on a Mono Q HR 5/5 column. Protein, 0.2 mg (in 50 mM Tris HCl buffer, pH 8.5), was applied to the column, which was equilibrated with the same buffer. The pullulanase was eluted with an NaCl gradient at a flow rate of 1 ml/min.

TABLE 1. Purification of the cloned pullulanase of *P. woesei* after expression in *E. coli*[a]

Step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Recovery (%)
Cell extract	1,530	230	0.15	100
Heat treatment	240	180	0.75	79
Maltotriose-Sepharose[b]	9.2	80	8.6	34
Mono Q	2.5	34	13.5	15

[a] After the aerobic growth of *E. coli* at 37°C, a 4-liter culture was centrifuged (16 g of cells, wet weight) and cell extract was prepared after sonication. Cell extracts containing 1,530 mg of protein (230 U of pullulanase) were boiled for 20 min.

[b] Affinity chromatography on a maltotriose-coupled Sepharose 6B column.

pullulanase of *P. woesei* is composed of a single polypeptide chain with a molecular mass of 90 kDa.

The pullulanase showed activity over a broad pH range, with more than 70% activity between pH 5.5 and 8.0. The pH optimum was determined to be 6.0 (Fig. 3a). The temperature optimum of the purified enzyme is 100°C, and a rapid decrease in pullulanase activity was observed above this point (Fig. 3b). An Arrhenius plot shows linearity between 50 and 100°C and evidence for an activation energy of 39 kJ/mol (Fig. 3b, inset).

No loss of enzymatic activity was observed after incubation of the purified pullulanase at 90°C for 4 h. After incubation at 100°C for 4 h, the residual activity was 85%. A substantial loss of activity was observed at 110°C (Fig. 4). The half-life of the enzyme at 110°C in the presence of maltodextrin (0.25%) was raised from 7 to 20 min. The same result was obtained when 0.25% maltodextrin was replaced by 1 mM Ca^{2+}. The thermostability of pullulanase was not influenced by the addition of pullulan (0.25%).

Kinetic experiments were performed by using the standard activity assay, with pullulan, soluble starch, and maltodextrin as substrates. K_m and V_{max} values were obtained from Lineweaver-Burk plots, using two concentrations of pullulanase (0.02 and 0.07 mg/ml). The enzyme followed Michaelis-Menten kinetics with all substrates tested; the enzyme had the

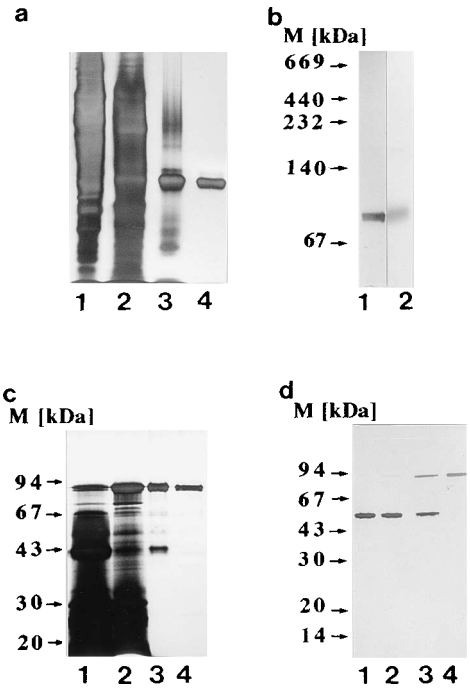

FIG. 2. Gel electrophoretic analysis of samples from various purification steps. Proteins were detected by silver staining. (a) Native PAGE. Lane 1, crude extract (10 μg of protein); lane 2, crude extract after heat treatment (10 μg of protein); lane 3, affinity chromatography pool (3 μg of protein); lane 4, Mono Q pool (0.4 μg). (b) Native polyacrylamide gradient gels (5 to 27%). Lane 1, purified pullulanase (0.4 μg); lane 2, zymogram. (c) SDS-PAGE. Lane 1, crude extract (10 μg); lane 2, crude extract after heat treatment (10 μg); lane 3, affinity chromatography pool (3 μg); lane 4, Mono Q pool (0.4 μg). (d) Influence of temperature on migration of the purified pullulanase in SDS gels. Samples (0.15 μg of protein per lane) were incubated for 10 min at 4°C (lane 1), 60°C (lane 2), 80°C (lane 3), and 100°C (lane 4).

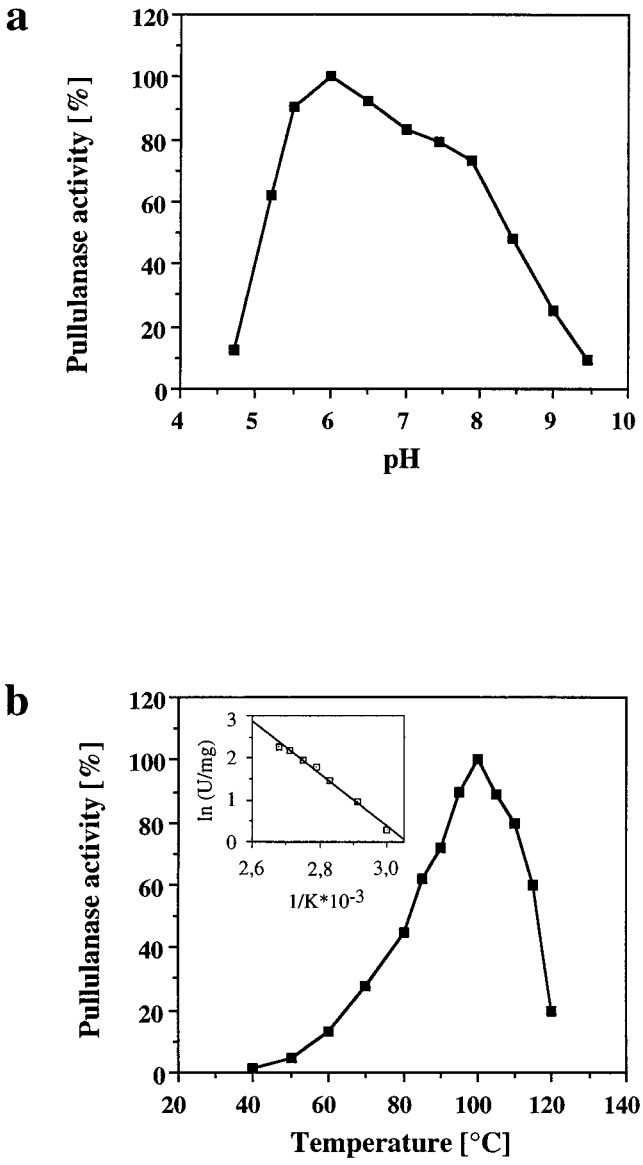

FIG. 3. Influence of pH (a) and temperature (b) on activity of the cloned pullulanase from *P. woesei*. The purified enzyme (0.02 mg/ml) was incubated at different pHs (in 50 mM MES-BisTrisPropane buffer, pH 4 to 6.5; and in 50 mM BisTrisPropane-MES buffer, pH 7 to 10) and at different temperatures (in 50 mM Na acetate buffer) for 5 and 10 min, respectively. For determination of temperature optimum, the purified enzyme (0.02 mg/ml) was incubated in Na acetate buffer (pH 5.5) and incubation was performed for 10 min at various temperatures (40 to 120°C). (Inset) Arrhenius plot showing linearity between 50 and 100°C.

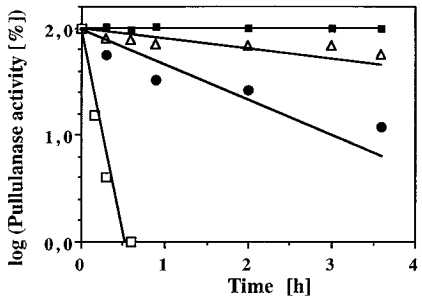

FIG. 4. Thermostability of the pullulanase from *P. woesei*. The purified enzyme (0.3 U/ml) was incubated at 90°C (■), 100°C (△), 110°C (□), and 110°C with 0.25% maltodextrin (●). Samples were withdrawn and tested for pullulanase activity at 90°C.

highest affinity with pullulan. The K_m for pullulan was determined to be 1.3 mg/ml; that for soluble starch was 2.5 mg/ml, and that for maltodextrin was 5 mg/ml. From the Lineweaver-Burk plot, the V_{max} values for pullulanase were calculated to be 35 U/mg for pullulan, 15 U/mg for starch, and 3 U/mg for maltodextrin; the standard deviation was less than 10%.

Substrate specificity and analysis of hydrolysis products. The hydrolysis pattern after the action of pullulanase on pullulan revealed the complete conversion of pullulan to maltotriose. More than 98% of pullulan was hydrolyzed after 1 h of incubation at 90°C (Fig. 5a). In order to confirm that the hydrolysis product from pullulan was maltotriose (possessing two α-1,4-glycosidic linkages) and not panose or isopanose (possessing α-1,4- and α-1,6-glycosidic linkages), incubation was also performed in the presence of α-glucosidase from yeast cells. The formation of glucose as the main product (Fig. 5g) confirmed the formation of maltotriose (not panose) from pullulan. Other branched oligosaccharides such as starch, maltodextrin, and glycogen, which contain α-1,6- as well as α-1,4-linkages, were also hydrolyzed by the pullulanase, resulting in oligosaccharides with a chain length ranging from 1 to 6 (Fig. 5b to d). Accordingly, the enzyme attacks α-1,6- as well as α-1,4-glycosidic linkages and can be classified as pullulanase of type II (or amylopullulanase). In addition to α-1,6-linkages in pullulan, the enzyme was also able to attack α-1,4-linkages in amylose, but at a lower rate (Fig. 5e). The first soluble product was DP6, which could be analyzed after 10 min of incubation (not shown). Incubation for 2 h caused the hydrolysis of DP6 to short-chain oligosaccharides ranging from DP1 to DP5. The α-1,6-linkage in panose was hydrolyzed at a very slow rate, and approximately 2% of the substrate was converted to maltose and glucose after 3 days of incubation at 90°C (Fig. 5f). Dextran, which contains exclusively α-1,6-linkages, was not hydrolyzed by the purified enzyme.

Influence of metal ions and other reagents. Pullulanase activity was not influenced by the addition of various metal ions such as Na^+, Mn^{2+}, Mg^{2+}, and Co^{2+} (Table 2). A pronounced effect was observed with $CaCl_2$. The addition of 0.3 mM Ca^{2+} caused an increase of enzymatic activity to 360%. Ni^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, and Cr^{2+} caused strong inhibition of the enzyme. Incubation of the purified pullulanase with 5 mM SDS and 6 M urea caused decreases in activity to 13 and 50%, respectively (Table 3). Activation up to 200 and 150% could be observed by incubating the enzyme with reducing agents such as β-mercaptoethanol and dithiothreitol, respectively. Triton X-100 (0.1 and 1%) also caused activation of the enzyme. Iodoacetamide (up to 10 mM) had no significant effect on the activity, while with 0.01% *N*-bromosuccinimide (NBS) the loss of activity was complete (Table 3). Unlike β- and γ-cyclodextrins, the addition of 0.1% α-cyclodextrin caused strong inhibition of pullulanase activity. None of these cyclic compounds were attacked by the enzyme. α-Cyclodextrin was not inhibitory when pullulan was replaced by starch or amylose in the enzyme assay. Under these conditions and in the presence of 0.1% α-cyclodextrin, more than 70% of enzymatic activity (amylolytic activity) was measured (Table 4). A similar effect was observed when NBS was added at a concentration of 0.01%. Similar to α-cyclodextrin, NBS inhibited the α-1,6-hydrolyzing activity (pullulytic activity) and not the α-1,4-hydrolyzing activity (the amylolytic activity). Specific inhibition of the α-1,6-hydrolyzing activity by these reagents seems to be possible.

Fluorescence spectroscopy. The emission spectra of the purified pullulanase at temperatures from 37 to 90°C showed strong temperature dependence concerning the quantum yield. The fluorescence emission of the enzyme exhibits a maximum at 339 nm (Fig. 6a). No shift in the maximum could be observed under these conditions. The emission spectra changed dramatically when the enzyme was incubated at 120°C. In this case, a new maximum arises at 307 nm. Incubation of pullulanase with SDS (5 mM) also caused significant changes in the spectra (Fig. 6b). After 2 min of incubation at 90°C with 5 mM SDS, a shoulder at 307 nm becomes apparent. Further incubation for 10 min leads to the formation of a new peak at 307 nm. After the sample is boiled for 5 min, the first peak with the 339-nm maximum disappears and a second peak with the 307-nm maximum is observed.

DISCUSSION

The enzyme system of *P. woesei* which is involved in the bioconversion of starch to glucose is composed of a number of extracellular and intracellular enzymes. These include an extracellular α-amylase, cell-associated pullulanase, intracellular α-amylase, and α-glucosidase (27, 32, 34). Gel electrophoretic analysis indicates that the pullulytic enzyme system is also composed of more than one protein (7). It is unclear, however, whether this phenomenon is due to expression of various pullulanase genes or to protein modification. From the peak form which resulted from laser desorption by matrix-assisted laser desorption mass spectrometry, it could be deduced that the purified pullulanase is not glycosylated. The enzymes from the very closely related organism *P. furiosus* and a few moderate thermophiles were reported to be glycosylated (8, 39, 49, 56). The majority of microbial pullulanases, however, including also extremely thermostable enzymes, seem to exist in an unmodified form. At high temperatures covalently bound carbohydrates may account for the stabilizing effects (17), but they do not seem to be essential for thermostability. This was confirmed for a number of cloned thermostable enzymes (46).

This is the first report on cloning of a gene encoding a polymer-degrading enzyme from a hyperthermophile. Around 1% of the total *E. coli* protein represents the *P. woesei* pullulanase. The cloned pullulanase is characterized by temperature and pH optima comparable to those of the enzyme from *P. woesei*. The pH range of 5.5 to 6 is very common for pullulan-hydrolyzing enzymes from various microorganisms such as *Thermococcus litoralis*, *P. furiosus*, *Bacillus* strains (*B. polymyxa* and *B. stearothermophilus*), *Thermus* sp., and *Clostridium* sp. (8, 10, 18, 29, 42, 45). The temperature optimum of the pullulanase is in the temperature range of the optimal growth temperature of *P. woesei* (59) as reported for several intra- and extracellular glycolytic enzymes from *Pyrococcus* sp. (8, 12, 23, 27), but these enzymes differ considerably in thermostability. The purified pullulanase also shows high thermostability in this

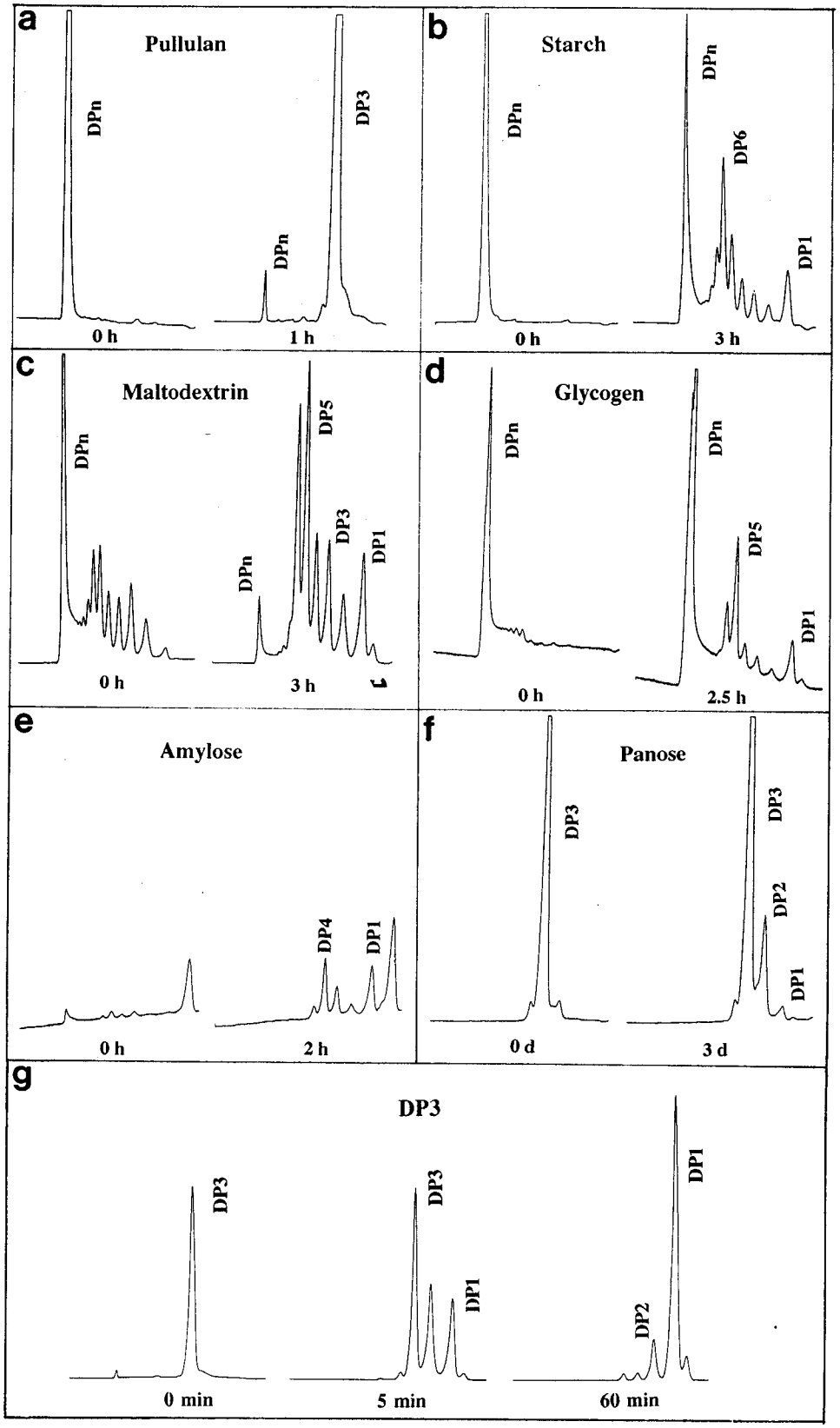

FIG. 5. HPLC analysis of hydrolysis products formed after incubation of the purified pullulanase (1.2 U/ml) with 1% polysaccharides (a to e) or 0.5% panose (f). Samples were incubated at 90°C, and at different time intervals aliquots were withdrawn and analyzed on an Aminex HPX 42-A column for oligosaccharides. The product formed after pullulan hydrolysis was incubated with commercial α-glucosidase and then analyzed by HPLC (g). DP, Degree of polymerization. DP1, glucose; DP2, maltose; etc.

TABLE 2. Influence of metal ions on pullulanase activity

Metal ion	Relative pullulanase activity (%)		
	1 mM	3 mM	5 mM
EDTA	100		
NaCl	96	103	95
$MnCl_2$	126	106	99
$MgCl_2$	128	103	99
$CaCl_2$	363	370	360
$CoCl_2$	135	105	94
$NiCl_2$	90	71	44
Na_2Mo_4	120	115	127
Na_2WO_4	121	133	110
$CuCl_2$	0	0	0
$ZnCl_2$	0	0	0
$FeSO_4$	0	0	0
$CrCl_3$	0	0	0

temperature range. Above this temperature, however, the enzyme is rapidly inactivated ($t_{1/2}$ = 7 min at 110°C).

From gel electrophoretic and spectroscopic studies, it is apparent that conformational changes accompany activation and thermal denaturation of the enzyme. Thermal denaturation is accomplished under extensive denaturation at 120°C for 20 min. The apparent molecular mass of the enzyme is 90 kDa. This value was also obtained by the matrix-assisted laser desorption mass spectrometry method. When the enzyme is incubated below 60°C, it shows a single band with an apparent molecular mass of 45 kDa in SDS gels. The fluorescence spectra also indicate that under these conditions the protein is very rigid, and its fluidity and activity increase upon elevation of the temperature to 100°C. Alteration of the protein conformation does not seem to be a continuous process. The enzyme seems to switch from the extreme compact form (45 kDa) to the unfolded denatured form (90 kDa). The extraordinary resistance of archeal enzymes against denaturation was also reported for various enzymes from hyperthermophiles, such as β-glucosidase from *P. furiosus* and protease from *Thermococcus stetteri* (23, 25).

TABLE 3. Influence of chemical reagents on pullulanase activity[a]

Reagent	Concn	Pullulanase activity (%)
SDS	1 mM	100
	5 mM	13
Urea	3 M	100
	6 M	50
β-Mercaptoethanol	10 mM	170
	30 mM	145
Dithiothreitol	10 mM	145
Iodoacetamide	10 mM	62
	20 mM	59
Triton X-100	0.1%	210
	1.0%	202
Guanidine HCl	0.25 M	54
	0.5 M	23
NBS	0.01%	0
α-Cyclodextrin	0.1%	8
β-Cyclodextrin	0.1%	69
γ-Cyclodextrin	0.1%	75

[a] Purified pullulanase was dialyzed against 50 mM Na acetate buffer (pH 5.5). Samples (0.3 U/ml, final concentration) were then preincubated with either metal ions or reagents at 90°C for 15 min. Aliquots were tested for pullulanase activity by incubating the samples with 0.5% pullulan in 50 mM Na acetate buffer at 90°C for 10 and 20 min, respectively.

TABLE 4. Influence of NBS and α-cyclodextrin on pullulytic and amylolytic activities of the purified enzyme (amylopullulanase)[a]

Reagent	Pullulytic activity (%; pullulan)	Amylolytic activity (%)	
		Starch	Amylose
NBS (0.01%)	0	60	80
α-Cyclodextrin (0.1%)	8	90	70

[a] Purified pullulanase was dialyzed against 50 mM Na acetate buffer (pH 5.5). Samples (0.3 U/ml, final concentration) were then preincubated with reagents at 90°C for 15 min. Aliquots were tested for pullulanase activity by incubating the samples with 0.5% pullulan in 50 mM Na acetate buffer at 90°C for 10 and 20 min, respectively. The amylolytic activity was determined with 0.5% starch and 0.5% amylose in 50 mM Na-acetate buffer (pH 5.5) as substrates.

Although the purified pullulanase is active without any metal ions, the enzyme is slightly stabilized and activated with Ca^{2+}. Similar effects have been observed with the pullulanases from *Thermus* sp., *Clostridium thermohydrosulfuricum* (40, 42), and *B. stearothermophilus* (30).

Reducing agents such as β-mercaptoethanol and dithiothreitol cause a significant activation of the cloned pullulanase and indicate that thiol groups are necessary for enzyme activity. Furthermore, the strong inhibition of the enzyme by NBS, a specific tryptophan-oxidizing agent (54), suggests the involvement of tryptophan residues in the catalysis. A similar effect was also reported for pullulanases from *Thermus* sp. and *B. stearothermophilus* (22, 42). Although the α-1,6 hydrolyzing

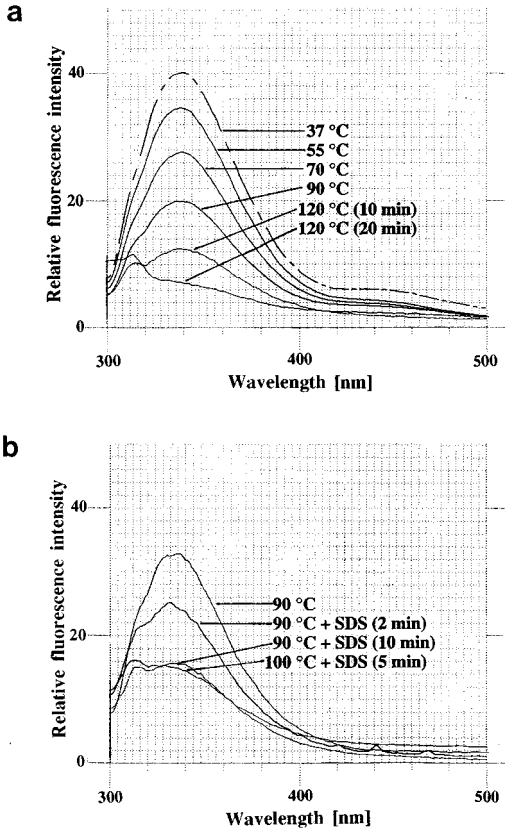

FIG. 6. Fluorescence spectra of purified pullulanase (0.02 mg/ml). (a) Emission was monitored at 37, 55, 70, 90, and 120°C. Incubation at 120°C was performed in an oil bath for 10 and 20 min, and the fluorescence was measured subsequently at 90°C. (b) Fluorescence emission of the pullulanase in the presence of 5 mM SDS after incubation at 90 and 100°C.

activity is totally abolished by NBS and by α-cyclodextrins, the pullulanase still hydrolyzes α-1,4-linkages in amylose and starch even in the presence of these inhibitors. One can speculate that the dual substrate specificity of the pullulanase is due to the presence of two active sites. The pullulanases from *Thermoanaerobium* strain Tok6-B1 and *C. thermohydrosulfuricum*, on the other hand, seem to possess only one active site (37, 44).

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