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Heat-stable pullulanase from *Bacillus acidopullulyticus*: characterization and refolding after guanidinium chloride-induced unfolding

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Abstract Heat-stable pullulanase from *Bacillus acidopullulyticus* was characterized with respect to its stability against thermal and chemical denaturation and its reactivation after complete chemical unfolding. The enzyme was quite thermostable and retained 55% of activity after heating at 60°C for 30 min at pH 5.5. At pH 6.0, only 9% residual activity was observed. The addition of sucrose, polyols, and Na_2SO_4 strongly stabilized the enzyme against thermal inactivation. The processes of chemical unfolding by guanidinium chloride (GdmCl) and refolding were studied by enzymological and spectroscopic criteria. *B. acidopullulyticus* pullulanase was very sensitive to GdmCl denaturation and had a transition midpoint at 1.2 M GdmCl. Reactivation after complete unfolding in 5 M GdmCl was initiated by dilution of the unfolding mixture; 67% reactivation was observed under standard conditions. The influence of some chemical and physical parameters (pH, chemical agents, temperature, and unfolding and refolding time) on refolding was investigated. Of the additives tested to assist reactivation, only bovine serum albumin (BSA) increased the yield of activity to 80%. The full regain of structure and activity was proven by comparing the enzymological, physicochemical, and spectroscopic properties of the native and refolded pullulanase.

Key words Pullulanase · *Bacillus acidopullulyticus* · Denaturation · Renaturation · Thermostability

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Introduction

Pullulanases (pullulan 6-glucanohydrolases, EC 3.2.1.41) catalyze the hydrolysis of α-1,6-linkages of pullulan and other branched polysaccharides. Pullulanases are monomeric proteins with molecular masses in the range 60–140 kDa (Vihinen and Møntsølø 1989). Many thermoactive pullulanases investigated so far attack both α-1,4 and α-1,6-glucosidic linkages in branched substrates, and these have been named pullulanase type II or amylopullulanase. The pullulanases that hydrolyze preferentially α-1,6-linkages (type I pullulanases) have been detected in the mesophilic bacteria *Klebsiella pneumoniae* (Bender and Wallenfels 1961), formerly classified as *Aerobacter aerogenes* (Vihinen and Møntsølø 1989), *Streptococcus mitis* (Walker 1968), *Bacillus sp.* (Nakamura et al. 1975), and in the thermophiles *Bacillus flavocaldarius* (Suzuki et al. 1991), *Thermoactinomyces thalpophilus* (Odibo and Obi 1988), *Fervidobacterium pennavorans* (Koch et al. 1997), and *Bacillus acidopullulyticus.* Pullulanase from *B. acidopullulyticus* has been purified and some physicochemical properties of the enzyme have been reported (Norman 1983; Schülein and Hojer-Pedersen 1984; Kusano et al. 1988; Lappalainen et al. 1991).

In the last decade the search for thermostable enzymes suitable for industrial applications led to the purification and characterization of thermostable pullulanases from various microorganisms (*Thermoanaerobium*, *Clostridium*, *Bacillus*, *Pyrococcus*, *Fervidobacterium*) (Plant et al. 1987; Saha et al. 1988; Spreinat and Antranikian 1990; Kuriki et al. 1988; Rüdiger et al. 1995; Koch et al. 1997). Recently genes encoding thermostable pullulanases have been cloned and expressed in mesophilic hosts (Kuriki et al. 1988; Rüdiger et al. 1995). The nucleotide sequence of the gene encoding *Bacillus stearothermophilus* enzyme has been reported (Kuriki et al. 1990). The extremely thermostable amylopullulanase from *Pyrococcus woesei* was the first enzyme of this class to be crystallized (Knapp et al. 1995).

Despite extensive investigations on thermostable pullulanases over the past years, no attention has been focused so

far on their structural stability and behavior in denaturant solutions and on the possibilities for recovering the biological functions after complete unfolding. The study of successful refolding strategies has become extremely challenging, and not only for basic research. The advent of recombinant DNA techniques raised the problem of inclusion body formation and the necessity of subsequent protein solubilization and reactivation. The aggregation of accumulating heterologous proteins has been observed in the majority of cases of eukaryotic gene expression in *E. coli* but also in other recombinant systems of microbial and animal host cells. High-level expression of wild-type protein can under certain circumstances also lead to inclusion body formation (Thatcher and Hitchcock 1994 and references herein). In recent years many communications have appeared reporting on the successful refolding of various oligomeric and monomeric proteins either using their ability for spontaneous regain of structure or by means of assisted refolding (Rozema and Gellman 1995; Solovikova et al. 1996; Tams and Welinder 1996).

This article presents the further characterization of *B. acidopullulyticus* pullulanase with respect to its conformational stability and reports for the first time on successful refolding of microbial pullulanase after complete chemical denaturation.

Materials and methods

Chemicals and buffers

Promozyme 200L from Novo Industri (Bagsvaerd, Denmark) was used as a source for purification of *B. acidopullulyticus* pullulanase. Guanidinium chloride, maltose, 1,4-dithio-threitol (DTT), EDTA, and $MgSO_4$ were obtained from Fluka BioChemica Microselect (Buchs, Switzerland); pullulan from Aureobasidium pullulans was from Sigma (St. Louis, MO, USA); Coomassie brilliant blue (CBB) R-250 and electrophoresis reagents were purchased from Bio-Rad (Richmond, CA, USA); sorbitol, bovine serum albumin, sucrose, and Na_2SO_4 were from Merck (Darmstadt, Germany); and glycine and glycerol were products of Carlo Erba (Milan, Italy). All other chemicals were of reagent grade. Buffers used were as follows: (A) 50mM sodium acetate, pH 5.5; (B) 20mM sodium phosphate, pH 7.0; and (C) 20mM sodium phosphate, pH 6.0, containing 0.1mM DTT. Buffers were filtered through a 0.45- or 0.2-μm membrane and carefully degassed before use.

Enzyme and protein assays

The enzyme activity was determined by measuring the release of reducing sugars from pullulan. Pullulanase was incubated at 60°C in buffer A with 0.5% pullulan. The reactions were stopped by cooling on ice, and the amounts of reducing sugars released were determined by the dinitrosalycilic acid method (Bernfeld 1955). One unit of enzyme activity was defined as the amount of enzyme required to catalyze the formation of 1μmol/min reducing sugars under these assay conditions. Maltose was used as a standard. Protein concentration was determined with Pierce Coomassie protein assay reagent (Rockford, IL, USA) with bovine γ-globulin as a standard. Kinetic parameters were calculated using a nonlinear regression data analysis program, EnzFitter (Leatherbarrow 1987). At least 6–7 data pairs were used for calculations.

Size-exclusion chromatography

Purification of Promozyme 200L was performed preparatively with a HiLoad16/60 Superdex 200 column (Pharmacia, Uppsala, Sweden). The column was equilibrated with buffer A, containing 100mM NaCl. The enzyme preparation was loaded and eluted from the column at 0.75ml/min. Elution was monitored at 226nm. Fractions containing pullulanase activity were pooled and used throughout all experiments. The molecular mass of the enzyme was determined by analytical gel filtration using a Superose 12 column (Pharmacia) eluted with buffer A made 150mM in NaCl and 10mM in 2-mercaptoethanol, using a Dionex gradient pump (Dionex, Sunnyvale, CA, USA) at 0.4ml/min. Elution was monitored at 226 and 280nm with a Kontron D430 computerized detector (Kontron Biotech Instruments, Milan, Italy). The column was calibrated with the following standards: aldolase from rabbit muscle (161kDa), bovine serum albumin (BSA) (66kDa), ovalbumin (45kDa), and cytochrome C (12kDa).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SDS-PAGE was performed according to Laemmli (1970) using 10% polyacrylamide gels. Protein samples were boiled for 5 min with 1% 2-mercaptoethanol and 1% SDS before loading the gel. Bio-Rad low molecular mass marker proteins were used as standards. Gels were stained with CBB R-250.

Equilibrium studies of GdmCl-induced unfolding

Pullulanase was incubated in various concentrations of GdmCl (0–6.4M in 20mM phosphate buffer, pH 6.0) at 22°C for 24h. Protein concentration was kept at 60μg/ml.

Denaturation/renaturation experiments

For renaturation experiments, unfolding was carried out by 2-h incubation of the protein at 22°C in 5M GdmCl dissolved in buffer B plus 1.9mM DTT and 1.9mM EDTA. Protein concentration in the unfolding mixture was 3.75 mg/ml. Renaturation was induced by 250-fold dilution of the unfolding mixture with buffer C. After 30min the reaction yield was determined. Control experiments were performed

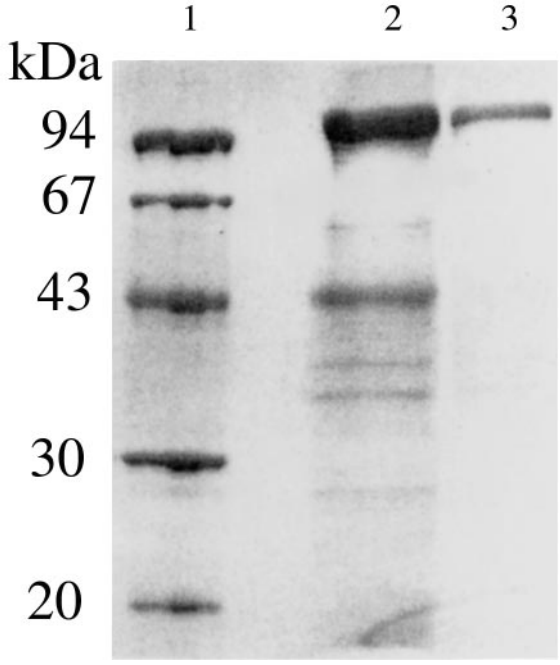

Fig. 1. SDS-PAGE of the purified *Bacillus acidopullulyticus* pullulanase. *Lane 1*, standard mixture; *lane 2*, commercial preparation (Promozyme 200 L) (12.5 μg); lane 3, purified enzyme (2 μg)

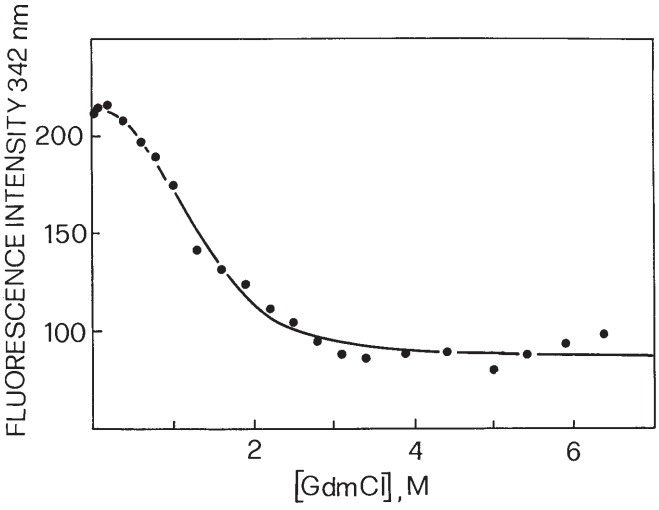

Fig. 2. Guandinium Chloride- (GdmCl-) induced denaturation of *B. acidopullulyticus* pullulanase. Fluorescence emission at 342 nm (excitation at 295 nm) was recorded at 22°C in the presence of increasing concentrations of GdmCl at 60 μg/ml protein concentration

with native enzyme that was treated under the same conditions but in the absence of denaturant.

Spectroscopic techniques

Fluorescence emission spectra were recorded on a Perkin Elmer (Norwalk, CT, USA) LS50B spectrofluorimeter at 22°C in a 1-cm cell. Excitation was at 295 nm; emission was recorded at 300–400 nm. Spectra were corrected for the solvent spectrum. Light scattering was followed on the same instrument with excitation and emission wavelengths were set at 400 or 480 nm.

Results

Enzyme properties

Pullulanase from *B. acidopullulyticus* was purified to homogeneity (Fig. 1, lane 3) from a commercial preparation (lane 2) (Promozyme 200 L, Novo Industri) by use of gel filtration chromatography. The purified enzyme was used in all experiments reported here. Native and denatured enzyme showed molecular mass of 97 kDa, demonstrating a monomeric structure. The native enzyme showed good thermostability and retained 55% of activity after incubation at 60°C for 30 min in buffer A. However, at pH 6.0 in 20 mM phosphate buffer, a dramatic decrease of activity to 9% with respect to the untreated control was observed after heating. Under these conditions the addition of 10% glycerol, 1 M sucrose, 20–30% sorbitol, and 1 M Na_2SO_4 prevented thermal inactivation of the protein, which was clearly demonstrated by the residual activities of 78%, 108%, 100%, and 101%, respectively. Calcium ions did not activate this pullulanase or protect it against thermal inactivation.

Protein concentration of *B. acidopullulyticus* pullulanase varied over the range 0.02–1.0 mg/ml did not affect the final residual activity after heat treatment. The purified enzyme remained fully active under storage at 4°C in buffer A and 0.02% NaN_3 for at least 6–8 weeks.

Unfolding

Equilibrium

Incubation of *B. acidopullulyticus* pullulanase at increasing concentrations of GdmCl for 24 h produced a progressive decrease of intrinsic fluorescence emission at 342 nm with a transition midpoint about 1.2 M GdmCl (Fig. 2). No increase in light scattering was detected during unfolding, compared to the native protein, indicating the absence of aggregated protein species. The intrinsic tryptophanyl fluorescence of the native *B. acidopullulyticus* pullulanase has its maximum at 342 nm, while that of the fully denatured protein was at 358 nm (Fig. 3).

Unfolding

To establish the time required for complete protein unfolding, pullulanase was incubated in 5 M GdmCl at 22°C. The fluorescence emission of the unfolding sample was recorded at increasing time intervals (from 20 min to 24 h of incubation). No change during this period was observed, indicating that the protein was totally unfolded in less than 20 min under these conditions.

From these results pullulanase unfolding was performed in 2 h in 5 M GdmCl in buffer B at 22°C in all following experiments. The presence of sulfhydryl group protective

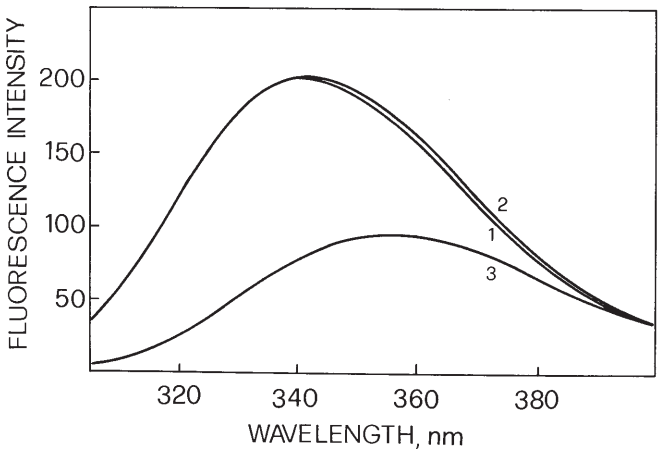

Fig. 3. Fluorescence emission spectra of native (*1*), refolded (*2*), and unfolded (in 5 M GdmCl) (*3*) pullulanase from *B. acidopullulyticus.* Excitation was at 295 nm. Spectra were recorded at 22°C; protein concentration was 60 μg/ml

Table 1. Effect of some additives on the yield of reactivation of *Bacillus acidopullulyticus* pullulanase after GdmCl-induced unfolding

Additive	Concentration	Percent of reactivation
None	–	67
$MgSO_4$	0.2 M	30
Sucrose	0.1 M	62
Pullulan	2.5%	55
Glycerol	10%	63
Na_2SO_4	0.4 M	13
Na_2SO_4	1.0 M	0
Bovine serum albumin (BSA)	10–50 μM	80
Sorbitol	25%	22
Sodium lauryl maltoside	0.5 M	37

GdmCl, guanidinium chloride

Table 2. Comparison between the catalytic and physicochemical properties of *B. acidopullulyticus* native and refolded pullulanases

Property	Native protein	Refolded protein
Specific activity (U/mg)	219	230
K_m for pullulan (mg/ml) (Lineweaver-Burk plots)	3.46	3.16
Temperature optimum	55°C	55°C
Ea (kJ/mol)	30.16	37.80

Ea, emergy of activation

agents such as DTT associated with EDTA is reported to be often required in the unfolding mixtures (Jaenicke and Rudolph 1989; Rozema and Gellman 1995). In the case of *B. acidopullulyticus* pullulanase, the molar ratio among protein, DTT, and EDTA was kept at 1:50:50.

Refolding

In the refolding experiments reported here, the unfolding mixture was diluted 250 fold in buffer C; the residual denaturant concentration was then 20 mM (and in the activity assay mixture was 4 mM, which was proven to be noninhibiting for the activity of the native enzyme). Samples were allowed to equilibrate in the refolding mixture for 30 min before the test of the enzyme activity.

The catalytic activity is considered to be a highly sensitive and specific test to monitor the proper folding of a protein because biological activity requires the three-dimensional structure of the protein to be in its native state (Jaenicke and Rudolph 1989). Complete renaturation of the protein was checked further by the comparison of fluorescence spectra with that of the native protein. When light scattering measurements of the refolding mixture showed the presence of aggregates from high protein concentration at unfolding (Jaenicke and Rudolph 1989; Maeda et al. 1996), these latter were removed by ultracentrifugation at 50 000 × *g* for 60 min. This procedure reduced the light scattering of the refolded protein solution to the control value (native protein). Refolding conditions were studied by varying the molar ratio protein:DTT:EDTA, in 20 mM phosphate buffer, pH 6.0. The best molar ratio for protein refolding was 1:700:50. Under these conditions, 67% reactivation was achieved. The increase of the DTT content in the refolding mixture up to 1:2700 (protein:DTT) did not influence the percentage of reactivation. A variety of conditions were tested to optimize the yield of reconstitution. Refolding in 100 mM acetate buffer pH 4.0, after unfolding in 5 M GdmCl in buffer A (pH 5.5), keeping constant all other parameters such as temperature and concentrations of denaturant, protein, and antioxidants, gave a reactivation of only 28%. As reported, unfolding was performed within 2 h, followed by equilibration in the refolding mixture for 30 min. The prolonged unfolding (up to 120 h) in 5 M GdmCl and refolding (overnight or longer) did not change the reactivation yield (67%).

The influence of various additives on the yield of reactivation is presented in Table 1. Only the addition of BSA to the refolding buffer assisted reactivation to some extent, yielding 80% of the original activity. The other additives tested, generally known as protein structure stabilizers, such as sugars, polyols, structure-stabilizing salts, and substrate (Timasheff and Arakawa 1989) did not improve the extent of protein renaturation. Additionally, the enzyme was completely inactivated by 2 M glycine and 1 M $MgSO_4$, concentrations at which these additives are usually used to stabilize the proteins (Timasheff and Arakawa 1989).

It is known that the rate of refolding generally varies with temperature in a complex manner: at low temperatures, the increase in temperature increases the rate of the process, while at high temperatures it decreases it (Creighton 1994). In *B. acidopullulyticus* pullulanase, refolding performed at 37°C yielded the same extent of reactivation as the control at 22°C, while the samples allowed to refold at 4°C showed 30% lower extent of reactivation compared to the control. A similar effect of temperature on refolding was observed by Zhi et al. (1992) in studies on citrate synthase. Attempts to carry out renaturation at 60°C failed because of protein instability or intermediate instability under these conditions. In Table 2 and Fig. 3, a compari-

son between the properties of native and refolded proteins is reported: refolded pullulanase shows no differences in K_m, optimal temperature, specific activity, and fluorescence spectra compared to the native protein.

Discussion

Purified *Bacillus acidopullulyticus* pullulanase is a monomeric protein with molecular mass of 97 kDa: the value is similar to those obtained for other pullulanases described so far (Vihinen and Møntsøløø 1989). Reports concerning Ca effect on *B. acidopullulyticus* pullulanase activity are controversial (Norman 1983; Lappalainen et al. 1991). We found that the enzyme was not stabilized by Ca^{2+}, nor was its activity enhanced by this cation, in contrast to other pullulanases of type I. For comparison, *Klebsiella pneumoniae* enzyme was activated by calcium ions (Ohba and Ueda 1975), while pullulanase from *Thermoactinomyces thalpophilus* had enhanced thermostability in the presence of calcium ions (Odibo and Obi 1988).

The enzyme shows good thermostability for a moderate extremophilic enzyme at pH 5.5, very close to its isoelectric point, 5.0–5.2 (Kusano et al. 1988; Lappalainen et al. 1991), where most of the proteins have maximal denaturation temperature (Stigter and Dill 1990). However, the enzyme thermostability is very sensitive to pH: increasing the pH to 6.0 is sufficient to obtain a dramatic decrease in enzyme thermostability. The maximal thermal stability close to the pI value may indicate that buried ionic groups of the protein are not important for protein stabilization. Some protective agents as glycerol, sucrose, and sorbitol, which decrease the interaction between protein and solvent, deleted the pH effect.

The native pullulanase shows a fluorescence maximum at 342 nm, indicating that the tryptophanyl residues are partially protected from the aqueous solvent (Schmid 1989). Guanidinium chloride-induced unfolding resulted in a red shift of the maximum of tryptophanyl fluorescence to 358 nm, caused by a full exposure of tryptophan side chains to the solvent. Refolding was obtained successfully by dilution of the unfolding mixture, but it is sensitive to pH and to the presence of antioxidants (EDTA and DTT). As a matter of fact, refolding was obtained with a 67% yield at pH 6.0 after unfolding at pH 7.0, whereas when the refolding process was carried out at pH 4.0, it was possible to reach a refolding efficiency of only 28% at pH 5.5. This finding confirmed the report of Jaenicke and Rudolph (1989) that isoelectric conditions must be avoided in denaturation/renaturation experiments.

Many authors have reported the successful assisted refolding of various proteins following complete denaturation (Rozema and Gellman 1995; Solovikova et al. 1996; Tams and Welinder 1996; Zhi et al. 1992). Of the additives tested, only bovine serum albumin (BSA) affected positively the refolding yield of *B. acidopullulyticus* pullulanase, 80% recovery of the original activity. Different explanations exist on the mechanisms of BSA-assisted refolding. BSA seems to cover the surfaces of vessels to which folding intermediates or unfolded proteins may stick (Jaenicke and Rudolph 1989; Lilie et al. 1993). Zhi et al. (1992) assumed that BSA and glycerol might employ a common mechanism by stabilizing structures that approach native conformation. However, with *B. acidopullulyticus* pullulanase, the yield of reactivation was unchanged in the presence of glycerol. Another explanation of the positive effect of BSA on refolding was proposed by Lilie et al. (1993): BSA, having hydrophobic pockets, may bind to hydrophobic regions of the proteins. This suggestion is supported by our finding that the reactivation yield was decreased at a lower temperature where the hydrophobic effect is expected to be reduced (Tandon and Horowitz 1989).

Additives that stabilize the native enzyme were effective in increasing the folding yield of reduced lysozyme in 4 M urea (Maeda et al. 1996). However, in the case of *B. acidopullulyticus* pullulanase, thermostabilizers such as glycerol, sorbitol, sucrose, and Na_2SO_4 did not enhance or even hamper protein renaturation. Obviously, they stabilized the folded protein, but did not assist refolding. Although many enzymes refold significantly in the presence of their substrates (Zhi et al. 1992), the addition of pullulan in saturating concentration to the refolding buffer was not very effective.

The complete regain of structure and activity was confirmed by comparing the enzymological and spectroscopic characteristics of both proteins, native and renatured. This result proves that refolding reactions of proteins are completely reversible if proper conditions are determined and that the amino acid sequence dictates the formation of a functional three-dimensional structure of the protein (Anfinsen 1973).

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