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Thermostability of β-xylosidase from *Aspergillus sydowii* MG49

Mousumi Ghosh, Geeta Nanda*

Department of Microbiology, Bose Institute, P-1/12, C.I.T. Scheme, VII – M, Calcutta – 700054, India

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Heating of *Aspergillus* β-xylosidase at 85°C ± 1°C and pH 5.5–6.0 (optimum for activity), causes irreversible, covalent thermoinactivation of the enzyme, involving oxidation of the thiol groups that are required for catalysis. Exogenous addition of cysteine, DTT, GSH and mercaptoethanol stabilizes the enzyme by extending its half-life. A similar effect is also exhibited by bivalent cations like Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+} and Zn^{2+} while, on the other hand Cu^{2+} accelerates thermoinactivation. Chemical modification of crude β-xylosidase with cross-linking agents like glutaraldehyde or covalent immobilization to a nonspecific protein like gelatin and BSA also enhances enzyme thermostability. These results suggest that addition of thiols and bivalent metal ions to a crude β-xylosidase preparation or immobilization/chemical modification enhances its thermal stability, thus preventing loss of catalytic activity at elevated temperatures.

β-Xylosidase; Thermostabilization; *Aspergillus sydowii*

1. INTRODUCTION

Investigations are carried out for producing catalytically active thermostable enzymes that are important industrially. This has the advantage of enhanced productivity, with less chance of microbial contamination, as well as an increased rate of substrate solubility, which ultimately leads to process efficiency. Enzymes become inactive at temperatures above a critical level due to unfolding of the molecules [1]. This process is usually reversible for most enzymes but prolonged heating results in irreversible loss of catalytic activity involving destruction of various covalent and noncovalent interactions [2]. Extensive work on the molecular mechanism underlying irreversible thermal inactivation was carried out by Klibanov et al. [3–5].

Earlier we reported the synthesis of an inducible β-xylosidase of intracellular origin from *Aspergillus sydowii* MG 49 [6], an enzyme constituting the xylanolytic system and responsible for hydrolysis of xylobiose to the pentose monomer xylose. In this work we have made an attempt to explain the probable mechanisms underlying thermoinactivation of crude β-xylosidase, from the same organism at a temperature of 85°C. The effect of enzyme cross-linking and immobilization on β-xylosidase thermostability was also determined.

*Corresponding author. Fax. (91) (33) 34 3886.

2. MATERIALS AND METHODS

2.1 *Microorganism*

Aspergillus sydowii MG 49 was isolated from decaying vegetation in our laboratory and maintained on 2% malt agar slants.

2.2. *Enzyme production*

Intracellular β-xylosidase was produced and extracted as described previously [6].

2.3. *Kinetics of irreversible thermoinactivation*

Crude *A. sydowii* β-xylosidase was suitably diluted and incubated at pH 6.0 (100 mM phosphate buffer) with/without various additives in a thermostatically controlled water bath at 85° ± 1°C. The time-course of enzyme inactivation was followed by periodically removing samples which were then cooled and assayed using *p*-nitrophenyl-β-D-xyloside as substrate at pH 6.0 and 60°C. The reaction mixture consisted of 100 μl each of enzyme, substrate (10 mM), and phosphate buffer (0.1 M, pH 6.0). The reaction was stopped by adding 1 ml 0.1 M Na_2CO_3 after incubating for 15 min. Liberated phenol was measured spectrophotometrically at 420 nm. Protein was measured by the method of Lowry et al. [7] standardized with bovine serum albumin.

One unit enzyme specific activity is defined as the amount of enzyme which produced one micromol of the product per minute, per milligram of protein, under the assay conditions.

2.4. *Chemical modification of β-xylosidase*

Crude β-xylosidase was treated with 2.5% solution of glutaraldehyde in 0.1 M sodium phosphate buffer, pH 6.0 for 18 h at 4°C and thereafter thermostability was measured and compared with that of the native enzyme.

2.5 *Immobilization of β-xylosidase*

Appropriately diluted crude β-xylosidase (10 ml, 9.2 $U \cdot mg^{-1}$ sp.act.) was covalently cross-linked, separately to gelatin and bovine serum albumin (10% w/v) with 2.5% (v/v) glutaraldehyde (diluted 10-fold with 0.1 M phosphate buffer, pH 6.0) and incubated overnight (~18 h) at 4°C. Thereafter, the matrix entrapped with the enzyme was washed with the same buffer and cut into small cubes (1 cm^3). Immobilized β-xylosidase was assayed as described above for the free en-

zyme and its thermostability at 85°C was compared with that of the native enzyme.

3. RESULTS AND DISCUSSION

3.1. *Kinetics of irreversible thermoinactivation of* Aspergillus sydowii β-*xylosidase*

Incubation of crude β-xylosidase at 85° ± 1°C and pH 6.0 (optimum for both activity and stability) resulted in a time-dependent loss of enzyme activity with a half-life of 18.5 min. Enzyme activity was not restored on cooling, indicating the irreversible nature of the inactivation. The time-course of β-xylosidase thermoinactivation did not follow first order denaturation kinetics. Instead it exhibited a sigmoidal rather than linear residual activation versus exposure-time curve (Fig. 1). The time-course showed a triphasic pattern with an initial slow rate followed by an increased rate and finally a slow rate before the completely denatured state of the protein was reached. The transition period varied with experimental conditions or additives incorporated. The initial slow rate of denaturation suggests a partially inactive state of the enzyme. The succeeding rapid rate indicates that various other processes cause thermodestruction in addition to that responsible for the partially inactive state. The main period of thermoinactivation showed first order kinetics as displayed by the linear region of the sigmoidal curve. Similar deviations from typical denaturation kinetics were also observed with endoglucanase I from *Trichoderma reesei* QM 9414 [8]. The reason for the decreased rate of inactivation before complete denaturation of the protein is not yet clear. Addition of reverse denaturants like urea, guanidine HCl and acrylamide did not stabilize β-xylosidase against heat inactivation (data not shown) indicating that conformational processes are not involved, since such agents not only disrupt non-covalent bonds but maintain the enzyme molecule in a highly unfolded state at elevated temperatures and thereby prevent the formation of incorrect structures on return to ambient temperature [3,9]. This gives an indication of the covalent nature of thermodestruction at 85°C.

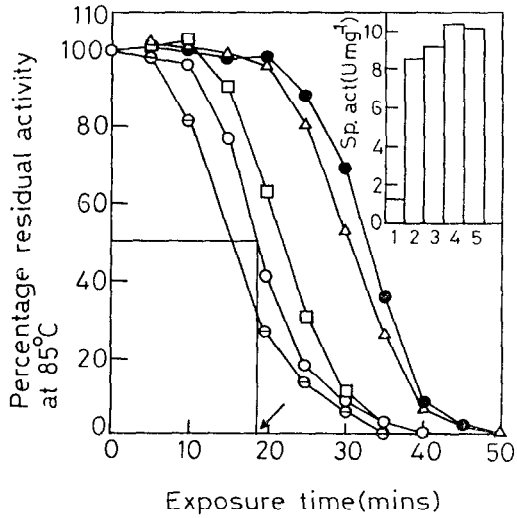

Fig. 1. Kinetics of irreversible thermoinactivation of crude β-xylosidase with and without added thiols at 85°C. pH 6.0 Native enzyme (○), native enzyme with 1 mM Cu^{2+} ions (⊖), 100 μM DTT (□), 1 mM GSH (△) and 1 mM cysteine HCl (●). Inset: Initial specific activity at zero min of native enzyme (2) with Cu^{2+} (1), DTT (3), GSH (4) and cysteine HCl (5).

3.2. *Effect of various additives on thermostability of β-xylosidase*

Addition of thiols, various bivalent cations and some detergents in low concentration increased enzyme thermostability.

3.2.1. Effect of thiols

Incorporation of exogenous thiols like cysteine, mercaptoethanol or GSH (Glutathione reduced) along with β-xylosidase increased enzyme thermostability by extending its half-life (Fig. 1). This indicates that sulfhydryl groups, essential for catalytic activity of β-xylosidase, are probably oxidized on heating. In order to investigate whether oxidation of cysteine residues is a primary cause of enzyme inactivation at high temperatures, thermostability was examined under similar conditions in the presence of either 100 μM DTT (dithiothreitol) as a reducing agent or 1 mM Cu^{2+} ($CuSO_4 \cdot 5H_2O$). The results in Fig. 1 show that the addition of DTT results in significant stabilization of the enzyme. In contrast, the addition of Cu^{2+} accelerated enzyme inactivation presumably by catalyzing air oxidation of thiols. Since a crude preparation of β-xylosidase was being tested, the actual number of thiol groups being oxidized in thermoinactivated samples of β-xylosidase could not be measured accurately. The requirement of cysteine residues for the catalytic activity of β-xylosidase was confirmed by treating the enzyme with 5 mM NEM (*N*-ethylene maleimide), a thiol group inhibitor, at 30°C. Only 40% residual activity remained after 10 min of incubation (data not presented). This suggests that the enzyme requires a free thiol for full activity. Hence, thermally induced oxidation of cysteines could be reversed/prevented on addition of thiols or reducing agents, thereby retarding enzyme inactivation.

3.2.2. Effect of bivalent cations

Addition of bivalent metal ions like Mg^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} at 1 mM concentration stabilized β-xylosidase at high temperatures (Table II). However, monovalent cations (Na^+, K^+) did not exhibit such an effect which is in contrast to the stabilizing effects of Hofmeister lyotropic series on irreversible thermoinactivation of enzymes [10]. Co^{2+}, Ca^{2+} and Zn^{2+} activated the enzyme to some extent at room temperature [6], as well as conferring stability at 85°C. Enzyme activity is maximally

reconstituted in the presence of Mg^{2+} and Mn^{2+}. Ca^{2+} has earlier been reported as a known thermostabilizing agent of various enzymes [11–13]. Surprisingly, except for Cu^{2+}, metal ions like Zn^{2+}, Mn^{2+} and Co^{2+} exhibited no inhibitory effect on activity/stability in spite of the requirement of a free thiol for β-xylosidase activity. Probably bivalent metal ions react nonspecifically with the protein-hydrophobic groups, resulting in salting out of these residues from the surface into the interior of the enzyme macromolecule as suggested earlier by Klibanov [2]. This compresses the enzyme conformationally resulting in high resistance to thermal unfolding and in greater thermal stability. The protective capacity of bivalent metal ions may be utilized for preparing catalytically active enzyme preparations of high thermostability.

3.2.3. Effect of detergents

Although detergents, whether ionic or nonionic, are known to be effective protein denaturants, surprisingly sodium taurocholate at 0.001% (w/v) final concentration increased β-xylosidase stability (Table I). Other detergents like SDS, sodium deoxycholate, Tween 80 and Triton X-100 did not show this effect. Protection of proteins from thermal denaturation by detergents at very low concentration was also reported by Lapanje et al. [1].

3.3. *Chemical modification of β-xylosidase*

Chemically modified crude β-xylosidase obtained after intra- and intermolecular covalent cross-linking on treatment with glutaraldehyde, exhibited greater thermostability than than of the native enzyme (Fig. 2). This may be due to protection of catalytic sites. Alternatively, the formation of thermoinactive conformations may have been prevented. Thermal stabilization by cross-linking was reported earlier with glutaraldehyde-modified β-glucosidase molecules [14].

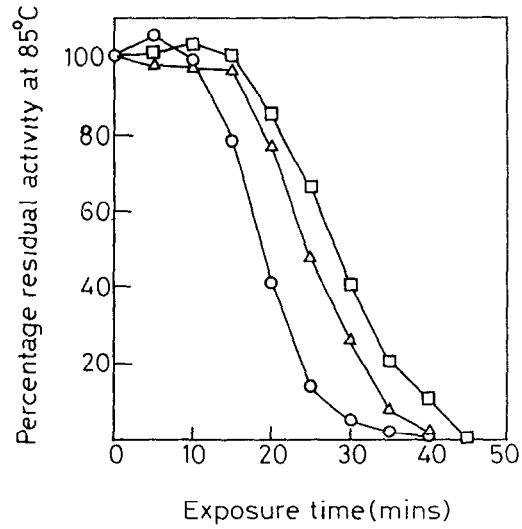

Fig 2. Comparison of thermostabilities of native (○), glutaraldehyde-treated (△) and mobilized β-xylosidase (□), cross-linked to gelatin.

3.4. *β-xylosidase immobilization*

Although immobilizing β-xylosidase to a nonspecific protein like gelatin or bovine serum albumin is accompanied by a loss of activity (35%, data not given), much higher thermoresistance was observed with the immobilized enzyme to its native counterpart (Fig. 2). This can be interpreted as a result of rigidification of the protein due to strong covalent attachment of the enzyme with the matrix, which in turn prevents unfolding and loss of

Table I

Effect of various detergents on thermostability of *Aspergillus sydowii* β-xylosidase

Detergent added to crude β-xylosidase (0.001% final conc.)	Percentage residual activity after incubating for 40 min at:	
	30°C	85°C
Control (enzyme without any detergent)	100.0	100.0
Sodium dodecyl sulphate	85.7	30.4
Sodium deoxycholate	101.4	65.2
Sodium taurocholate	107.1	121.7
Tween 80	101.3	100.0
Triton X-100	100.0	102.0

*Enzyme was preincubated with the detergent at a final concentration of 0.001% in sodium phosphate buffer (0.1 M, pH 6.0) for 30 min at room temperature. 100% activity of native enzyme incubated for 40 min at 30°C and 85°C corresponds to 8.6 and 0.37 U · mg $^{-1}$ sp.act., respectively.

Table II

Effect of various metal ions on thermostability of β-xylosidase

Metal ions added to β-xylosidase* (1 mM final conc.)	Percentage residual activity
Control (no metal ion added to enzyme)	100
Na^{+}	100
K^{+}	105
Ca^{2+}	132
Mg^{2+}	158
Zn^{2+}	147.5
Mn^{2+}	154
Cu^{2+}	27
Co^{2+}	150

*Enzyme was preincubated with different metal ions at room temperature for 30 min before exposing at 85°C for 20 min. 100% sp.act. of native enzyme after 20 min exposure at 85°C corresponds to 4.2 U·mg^{-1}.

three-dimensional structure of the protein on exposure to heat [15]. Immobilization of β-xylosidase would thus help in conservation of its catalytic activity even at high temperatures.

4. CONCLUSION

The thermostable β-xylosidase of *A. sydowii* undergoes irreversible inactivation at 85°C due primarily to oxidation of cysteine residues that are essential for enzyme catalysis. The sigmoidal time-course of inactivation may result in part from other processes such as hydrolysis of peptide bonds or destruction of amino acid residues. Incorporation of bivalent cations was found to decrease the rate of enzyme inactivation. In addition, cross-linking of β-xylosidase significantly increased thermostability and thus its practical utility.

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