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Biochemical approaches of increasing thermostability of β-amylase from *Bacillus megaterium* B_6

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Abstract Studies on the irreversible thermoinactivation of β-amylase from *Bacillus megaterium* B_6 exposed to 60°C revealed that the deactivation mechanism probably results from the oxidation of thiols present at the active site of the enzyme. Several attempts were made to increase its thermostability, which indicated that Mn^{2+} played a key role in determining thermostability and partially reactivating the inactivated enzyme. Immobilization of β-amylase through gel-entrapment and covalent crosslinking brought about a remarkable increase in thermotolerance with about a 14-fold increase in catalytic half-life.

Key words: β-Amylase; Thermostabilization; Adsorption; Immobilization; Irreversible thermoinactivation

1. Introduction

Utilization of enzymes in industrial biochemical reactors often encounter the problem of thermal inactivation of the enzyme. Since an increase in temperature causes an increase in vibrating energy of the molecule and collision energy between molecules [1], at high temperature enzymes undergo partial unfolding by heat-induced disruption of non-covalent interactions [2]. Therefore, in order to define rational strategies for enhancement of enzyme thermostability, irreversible thermal denaturation processes must be elucidated [3]. Thermoinactivation of an enzyme can be described by the following mechanism [4]:

$$N \rightleftharpoons U \rightarrow I$$

where N is the native catalytically active enzyme, U is the partially unfolded enzyme and I is the irreversibly inactivated enzyme. Therefore, thermostabilization of an enzyme can be achieved by decreasing the rate constant (K) and altering the activation energy of the enzyme (E_a) to a desired value [5]. This might be accomplished by solvent engineering [5–7], immobilization [8,9], or site-directed mutagenesis by protein engineering [10].

Although extensive studies have been carried out on the thermoinactivation of α-amylase [3,5,9–11], very little is known about the kinetics of thermodenaturation of β-amylase. In this study, we attempted to determine the underlying mechanism of thermoinactivation of partially purified β-amylase from *Bacillus megaterium* B_6, and elucidated the effectiveness of some approaches leading to its increased thermotolerance, thus increasing its industrial applicability.

2. Materials and methods

2.1. Enzyme source

Bacillus megaterium B_6, a potent producer of extracellular β-amylase, was isolated from starchy wastes and maintained in starch-peptone medium at pH 7.0 [12]. The culture filtrate was ultrafiltered through a membrane with a 100 kDa cut off, followed by salting out with 80% $(NH_4)_2SO_4$. The enzyme was subjected to gel-filtration (Sephadex G-200) and the resulting enzyme, β-amylase, was used for subsequent experiments. Sodium phosphate buffer (50 mM, pH 6.9) was used throughout the preparation.

2.2. Enzyme assay

β-Amylolytic activity was determined by the method of Bernfeld [13]. The assay mixture (1 ml), containing an equal volume of enzyme diluted with 50 mM phosphate buffer, pH 6.9, and 1% (w/v) starch solution, was incubated at 60°C for 5 min. One unit of enzyme activity corresponds to the amount that liberated 1 μmol of maltose per min per ml under optimal assay conditions. The thermostability of β-amylase was monitored by incubating the enzyme for 30 min. Residual activities were measured after cooling.

2.3. Stabilisation of β-amylase against irreversible thermodenaturation

Solutions of enzyme in 50 mM phosphate buffer, pH 6.9, were incubated at 60°C in the presence or absence of various additives. Aliquots were periodically withdrawn, cooled in ice for 10 min and residual activities measured.

2.4. Adsorption of β-amylase on raw starch

Properly diluted β-amylase (1 ml) was adsorbed onto 100 mg raw starch [14]. The raw starch-adsorbed enzyme was exposed to 60°C for 0–80 min and cooled. The residual activities of the desorbed enzyme were measured.

2.5. Immobilization of β-amylase

The enzyme solution, immobilized on a 5% (w/v) bovine serum albumin (BSA) matrix, was covalently crosslinked with 0.625% (w/v) gluteraldehyde. The enzyme film was washed with buffer and cut into small blocks. Immobilized β-amylase was assayed as described above with reciprocal shaking (60 strokes min^{-1}, 4 cm amplitude) during incubation.

2.6. Reactivation of thermoinactivated β-amylase

Aliquots from β-amylase solution preincubated at 60°C were periodically withdrawn, cooled in the presence or absence of Mn^{2+} and/or β-mercaptoethanol for 10 min followed by the determination of residual activities.

2.7. CD spectra

CD spectra were recorded at 50 nm/min scanning speed. Properly centrifuged and filtered samples were analysed in 1 cm optical path length.

3. Results and discussion

3.1. Thermal denaturation kinetics

Preincubation of β-amylase at 60°C (temperature optima for enzyme activity) led to the loss of about 80% of its activity

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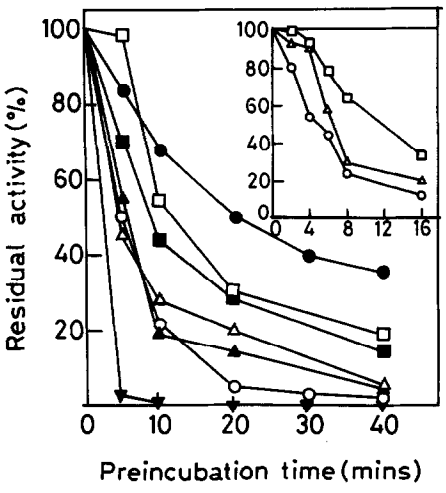

Fig. 1. A plot of the irreversible thermoinactivation kinetics of β-amylase preincubated at 60°C, pH 6.9, in the presence and absence of different additives. Native enzyme (○), native enzyme with: 4 mM Mn^{2+} (●), 1 mM β-mercaptoethanol (□), 1 mM cysteine (■), 1 mM reduced glutathione (△), 1 mM dithiothreitol (▲), and 1 mM Cu^{2+} (▼). Inset: effect of protein concentration on the thermostability of the enzyme protein: 0.05 mg/ml (○), 0.10 mg/ml (△), and 0.20 mg/ml (□).

within 10 min. The failure to recover the activity after returning to ambient temperature illustrated the irreversible nature of the thermo-inactivation [15]. The plot of enzyme denaturation kinetics (Fig. 1) was distinctly biphasic, with an initial rapid phase, obeying apparent first order kinetics, followed by a slower phase. Similar observations were found for α-amylase of *Bacillus licheniformis* [3] and glucose dehydrogenase of *Acinetobacter calcoaceticus* [16]. The CD spectrum of β-amylase (Fig. 2) indicated that thermal inactivation is associated with extensive denaturation and unfolding of the protein molecule. The rate of thermoinactivation was found to be decreased in proportion to the concentration of protein exposed, as evident from the extension of the half-life with increasing protein concentration (Fig. 1).

3.2. Effect of different additives on thermostability

Various additives, like metal ions, surfactants, exogenous thiols, polyols and proteins, were tested for their ability to increase the thermostability of β-amylase. The thermostabilization of β-amylase seemed to be Mn^{2+} specific (Table 1). Addition of Mn^{2+} (4 mM) brought about a fourfold increase in enzymatic half-life (Fig. 1), presumably by increasing the chemical potential of the protein and thereby favouring the folded state over the unfolded one: this was further supported by the fact that the accelerated thermoinactivation of β-amylase, in the presence of heavy metals (Cu^{2+}, Ag^{2+}, Hg^{2+}), could be partially overcome by Mn^{2+} ions (data not shown). Although bile salts and detergents (SDS) failed to improve the thermostability of β-amylase, the surfactants showed a striking ability to increase thermotolerance (Table 1).

Inactivation of β-amylase in the presence of thiol inhibitors (*p*-chloromercuribenzoate and *N*-ethylmaleimide) revealed the presence of thiol at the active site of the enzyme. Addition of exogenous thiols like cysteine, β-mercaptoethanol and reduced glutathione (GSH) prior to preincubation of β-amylase at 60°C, led to the extension of enzymatic half-life (Fig. 1). Increased thermostabilization of the enzyme in the presence of a

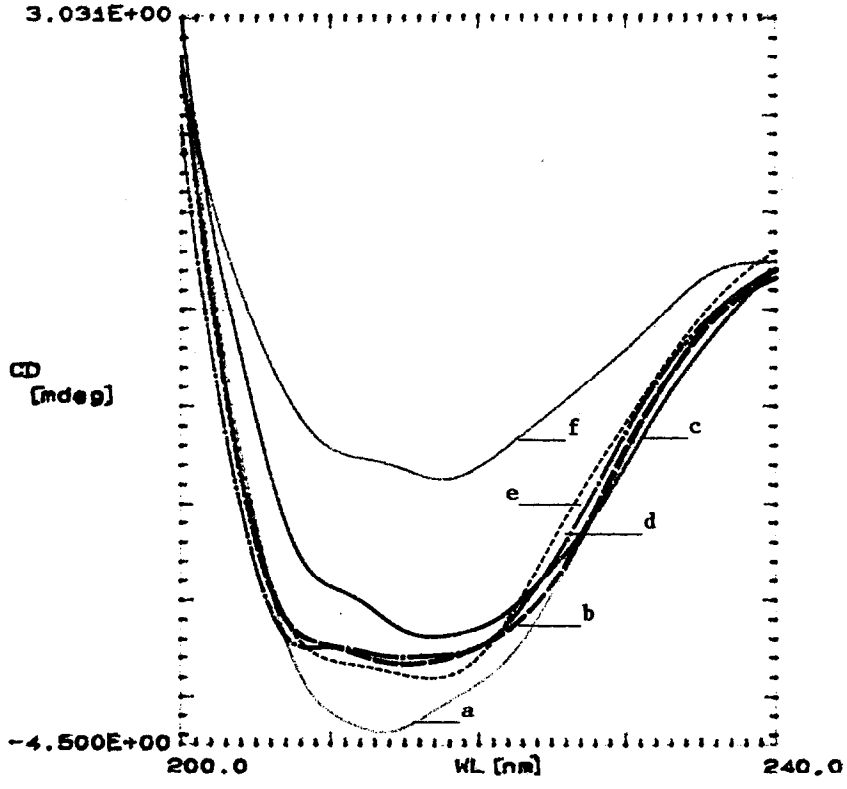

Fig. 2. The CD spectra of β-amylase after different periods of thermoinactivation at 60°C. (a) Native enzyme; (b) after 5 min, (c) 10 min, (d) 20 min, (e) 30 min; and (f) enzyme reactivated with Mn^{2+}.

reducing agent (dithiothreitol) and accelerated thermoinactivation in the presence of an oxidising agent (Cu^{2+}) indicated that thermoinactivation of β-amylase probably involved oxidation of thiols. A similar mechanism was found to be involved in the thermoinactivation of β-xylosidase of *Aspergillus sydowii* MG 49 [8].

3.3. Effect of enzyme immobilization on thermostability

β-Amylase was physically and chemically immobilized by adsorption and entrapment with covalent crosslinking, respectively. Raw starch-adsorbed β-amylase was found to be less susceptible to thermoinactivation in comparison to unbound enzyme (Fig. 3), which could be explained in terms of the conformational stability of the catalytic site imparted by the adsorbed raw starch. β-Amylase entrapped in a BSA matrix and crosslinked with gluteraldehyde exhibited increased thermostability compared to the native form, with a remarkable fourteenfold increase in catalytic half-life (Fig. 3). This enhanced thermotolerance was attributed to gel-entrapment and intramolecular crosslinking resulting in increased structural integrity of the protein molecule and less thermal exposure of the catalytic site.

3.4. Reactivation of thermoinactivated β-amylase

The thermoinactivated enzyme (exposed to 60°C for 10 min) could be partially reactivated by the addition of 1 mM β-mercaptoethanol (7.3% increase) and 4 mM Mn^{2+} (14% increase). An optimum combination of β-mercaptoethanol (0.5 mM) and Mn^{2+} (2 mM) was found to be able to revive of the inactivated β-amylase with an increase of about 43 ± 5% residual activity.

Table 1
Effect of various metal ions, bile salts and surfactants on the thermostability of β-amylase

Metal ions (1 mM)	Residual activity (%)	Surfactants and bile salts (0.001%)	Residual activity (%)
Control	100	Triton X-100	115
Na^{+}	114	Tween 40	120
K^{+}	99	Tween 80	101
Ba^{2+}	94	Sodium dodecyl sulfate	ND
Ca^{2+}	72		
Pb^{2+}	69	Sodium taurocholate	13
Sn^{2+}	102		
Co^{2+}	83	Sodium deoxycholate	ND
Mg^{2+}	96		
Cu^{2+}	4		
Mn^{2+}	194		
Bi^{2+}	107		
Sr^{2+}	75		
Zn^{2+}	68		
Ag^{2+}	5		
Fe^{3+}	138		

The enzyme was preincubated with additives (metal ions, bile salts or surfactants) for 20 min at 28°C followed by exposure to 60°C for 10 min. 100% activity refers to the activity of untreated native enzyme exposed to 60°C for 10 min. ND = not detected.

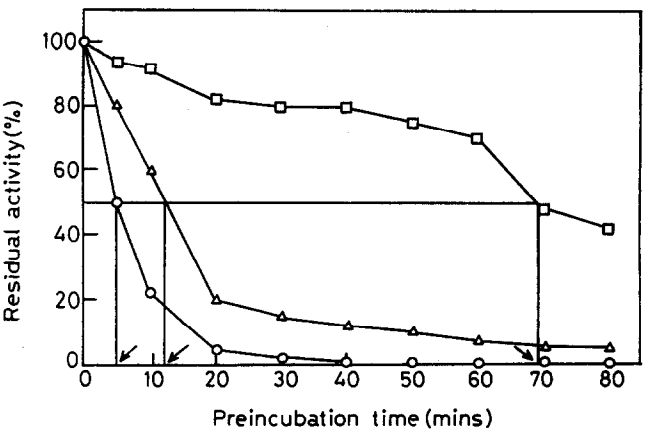

Fig. 3. Thermoinactivation kinetics of native enzyme (○), raw starch-adsorbed native enzyme (△), and covalently crosslinked gelatin-entrapped enzyme (□).

4. Conclusion

Increased thermoresistance of β-amylase was achieved through stabilization of thiol groups present at the active site by manipulating the enzyme's environment by the addition of Mn^{2+} and β-mercaptoethanol, and through immobilization; both of which would increase the enzyme's practical importance and industrial utility.

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