0273-2289/01/90/0047/$12.25

Comparative Studies on a Mesophilic and a Thermophilic α-Amylase

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Received November 1, 1999; Revised March 1, 2000; Accepted March 1, 2000

Abstract

A comparative study was performed on thermal stability of mesophilic and thermophilic α-amylases, and the effects of various denaturing agents, organic solvents, and stabilizers were investigated. As expected, the thermophilic enzyme showed higher resistance toward denaturation in water as its natural medium, but such a difference could not be detected in nonaqueous environments. Furthermore, stability of these molecules was improved by including various stabilizing agents. Of the compounds tested, sorbitol provided the highest degree of protection, which was found to be owing to its effect on increasing T_m and its ability in totally preventing deamidation of amino acid residues in the protein molecules.

Index Entries: α-Amylase; thermostability; deamidation; organic solvent; thermostabilizing additives.

Introduction

The stability of a protein during isolation and purification procedures is of vital importance in biotechnology and, accordingly, for its in vitro characterization *(1–3)*. Suggested mechanisms of protein denaturation together with the use of enzymes in organic solvents have provided much information that has been found quite useful in this connection *(4–7)*.

The concept of protein denaturation includes both reversible and irreversible changes *(8–10)*. The reversible process is a spontaneous event during which a protein reverts to its original state on removal of the denaturant. Reversibility may be attained using milder conditions (e.g., lowering the temperature). Heat, organic solvents, acid, detergents, urea, and so on may lead to denaturation of proteins by perturbing covalent and/or noncovalent

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interactions that are responsible for the maintenance of their native structures *(4,8–10)*.

Studies on thermophilic organisms are of theoretical and practical interest because proteins derived from these organisms are generally more stable than their mesophilic counterparts *(11–13)*. Thermozymes are defined as enzymes produced by thermophiles and hyperthermophiles that can function in the temperature range of 60 to above 110°C. A number of mesophiles also produce enzymes that are stable and active above 60°C. A particular type of α-amylase used in the present investigation is originated from *Bacillus licheniformis* (BLA), which is optimally active at 75–80°C. Mesophilic enzymes (or mesozymes) are enzymes that are optimally active at the moderate temperatures of 20–60°C *(11–13)*.

Stabilization of an enzyme may be achieved by the addition of different external factors. These compounds, known as stabilizing agents, are thought to enhance thermostability by modifying the structure of water or strengthening hydrophobic interactions inside protein molecules *(14–17)*. Dehydration of organic solvents surrounding enzymes hinders their inherent conformational mobility in water as their natural medium. Consequently, enhancement of thermostability is attained *(7,18–20)*.

In this study, the effects of extreme conditions such as high temperatures, low and high pH, organic solvents, chemical denaturing agents, and detergents on the stability of a thermophilic α-amylase obtained from BLA and its mesophilic counterpart extracted from *Bacillus amyloliquefaciens* (BAA) were investigated.

Materials and Methods

α-Amylase from BAA (Type II-A) was purchased from Sigma (St. Louis, MO) as a crystalline powder with a specific activity of 818 Sigma units/mg of protein. α-Amylase from BLA was generously provided by Novo. This enzyme was at least 95% pure as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Soluble potato starch, detergents, urea, Gdn-HCl, and trehalose were purchased from Sigma. The organic solvents were obtained from Merck (Darmstadt, Germany).

Determination of Enzymatic Activity

Activity was achieved colorimetrically at room temperature using soluble potato starch as substrate in 20 m*M* Tris buffer, pH 7.5. The concentration of reducing sugars obtained from the catalyzed reaction was measured by the dinitrosalicylic acid method according to Bernfeld *(21)*.

Determination of Thermal Stability

The time course of irreversible thermoinactivation of α-amylase was measured by incubating the enzyme (at a final concentration of 0.2 mg/mL) in 20 m*M* Tris buffer, pH 7.5, at the desired temperatures. The buffer was

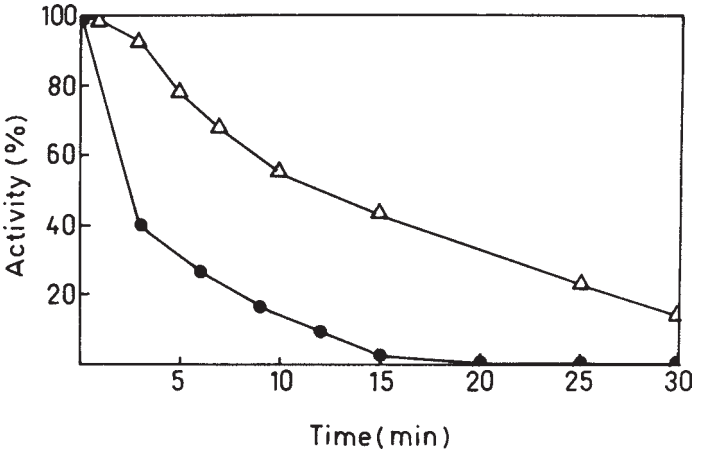

Fig. 1. Irreversible thermoinactivation of BLA (△) and BAA (●) at 95°C. Aqueous solution of each enzyme was incubated in 20 m*M* Tris, pH 7.5, at a final concentration of 0.2 mg/mL. At the indicated times, samples were drawn and activities determined. Further details are described under Materials and Methods.

adjusted to the suitable pH at the temperature of thermoinactivation experiments. At regular intervals, samples were removed and cooled on ice, and the remaining activity was determined as described above. Activity of the same enzyme solution kept on ice was considered as control (100%).

α-Amylases were also incubated at 80°C in the presence of glycerol, mannitol, sorbitol, and trehalose separately, each at a 20% concentration (v/v or w/v) in tightly closed tubes, and their effect on thermal stability was determined. Incubation times of 1 h and 30 min were chosen for the thermophilic and mesophilic enzymes, respectively.

Determination of T_m

T_m was determined by using solutions of the mesophilic or thermophilic enzymes at concentrations of 0.25 and 0.4 mg/mL of protein, which corresponded approximately to an optical density of 0.3 at 280 nm. A Gilford spectrophotometer was used, and the rate of increase in temperature was adjusted at 1°C/min.

Determination of Ammonia

Ammonia liberated in the deamidation reactions was determined using glutamate dehydrogenase and following a procedure essentially as described previously *(22)*. Tubes were tightly closed to avoid evaporation of ammonia.

Results

Thermal stabilities of BAA and BLA were compared at 95°C, pH 7.5. BLA was found to be considerably more stable than BAA under the conditions utilized (Fig. 1). The difference was especially pronounced at pH 7.0

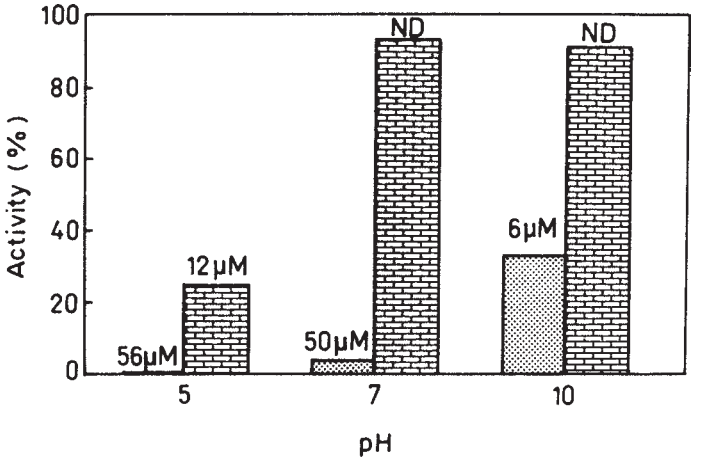

Fig. 2. Thermal stabilities of BLA (▦) and BAA (▭) at three different pH values. A 0.2 mg/mL solution of each enzyme was incubated in a mixed buffer containing acetate, Tris, and Caps, each at a 20 m*M* concentration and adjusted to the required pH. After incubation for 5 min at 95°C, activities were determined in the usual manner. Ammonia concentration corresponding to each pH run is also indicated for the two enzymes. ND, not detectable.

Table 1
Effect of Various Additives
on Irreversible Thermoinactivation of BLA and BAA at 80°C[a]

	None	Trehalose	Sorbitol	Mannitol	Glycerol
Remaining activity (%)					
BLA	70	79	95	93	90
BAA	4	7	20	14	9
Ammonia (μ*M*)					
BLA	23	ND	ND	11	9
BAA	35	ND	ND	13	12

[a]Enzyme solutions were prepared at a 0.2 mg/mL concentration in the presence of the additives (20%, w/v or v/v), and remaining activity was determined in the usual manner using incubation times of 1 h for the thermophilic enzyme and 30 min for the mesophilic protein. The extent of deamidation, presented as concentration of ammonia, was determined following a procedure described in Materials and Methods. ND, not detectable.

(Fig. 2). As indicated, deamidation was the lowest at the highest pH value tested.

α-Amylases were also incubated at 80°C in the presence of glycerol, mannitol, sorbitol, and trehalose separately, each at a 20% concentration (v/v or w/v) in tightly closed tubes, and the effect of these additives on thermal stability was investigated. As indicated in Table 1, all these additives brought about stability enhancement, with trehalose being the least effective. The T_m values for both enzymes were diminished by trehalose whereas all the other additives caused an increase in T_m (Table 2).

Table 2
Effect of Various Additives on T_m of BLA and BAA[a]

	None	Trehalose	Sorbitol	Mannitol	Glycerol
T_M					
BLA	83	80	>90	87	87
BAA	74	70	85	79	77

[a]Enzyme solutions were prepared in the presence of the additives at a 20% concentration (w/v or v/v) and the T_ms were determined. Further details are described in Materials and Methods.

To elucidate further the mechanism of denaturation, the extent of deamidation of amino acid residues in the proteins was determined. As shown in Table 1, all four additives caused lowering of deamidation, with sorbitol and trehalose being the most effective. Results presented on the effect of additives are the average of data from three experiments in a typical run. Experiments were repeated to confirm reproducibility.

Effect of Organic Solvents

Toluene and *n*-octane were chosen as two immiscible organic solvents, and their effects on the thermal stabilities of BAA and BLA were investigated. As indicated in Fig. 3, both enzymes were dramatically stabilized with no detectable loss of activity over the course of 3 h.

The effects of SDS as an anionic, DTAB (dodecyltrimethylammonium bromide) as a cationic, and SB-12 (lauryl sulfobetaine) as a zwitterionic detergent on catalytic potential of these enzymes were investigated by assaying for activity in the presence of various concentrations of these detergents at room temperature. There was little inactivation in the presence of the detergents, especially for BLA. In the case of BAA, the cationic and the anionic detergents caused up to 30% inactivation whereas the zwitterionic detergent (SB-12) had no effect, suggesting the importance of electrostatic interactions in the association of these detergents with the protein. When the same experiment was carried out at 60°C, more extensive inactivation was observed with greater differences in activity (Fig. 4). Again, the zwitterionic amphiphile was less effective than the other two detergents.

Urea and guanidine hydrochloride were also tested in this investigation using concentrations of 1, 2, 4, 6, and 8 *M* and 0.5, 1, 2, 4, and 6 *M* of the two chemical denaturants, respectively. For this purpose, the two enzymes were incubated separately with these agents at the specified concentrations at room temperature, and at various time intervals, samples were withdrawn and activities determined in the usual manner. In all cases, loss of activity was greater for the mesophilic enzyme in comparison with the thermophilic protein. Also, after 2 h of incubation, dialysis followed by activity determination indicated reversibility without exception.

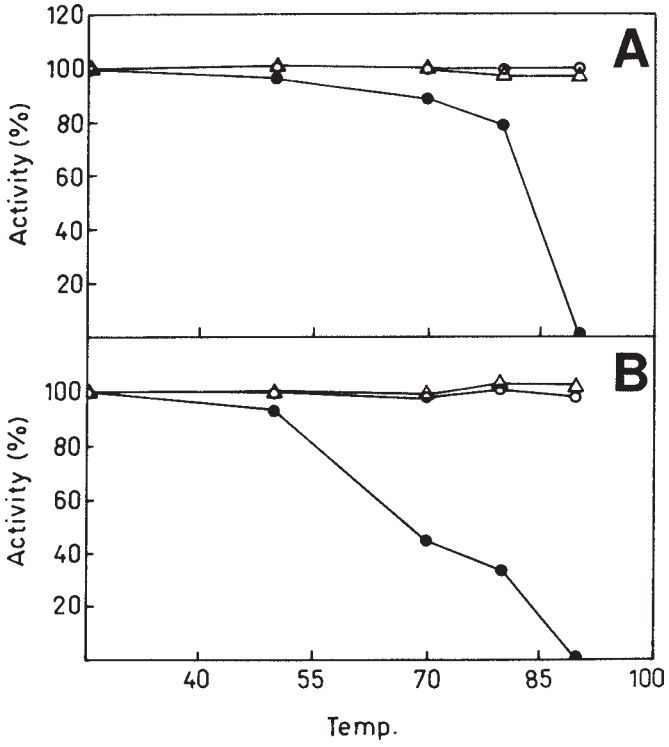
A
B
Activity (%)
120
100
80
60
40
20
40
55
70
85
100
Temp.

Fig. 3

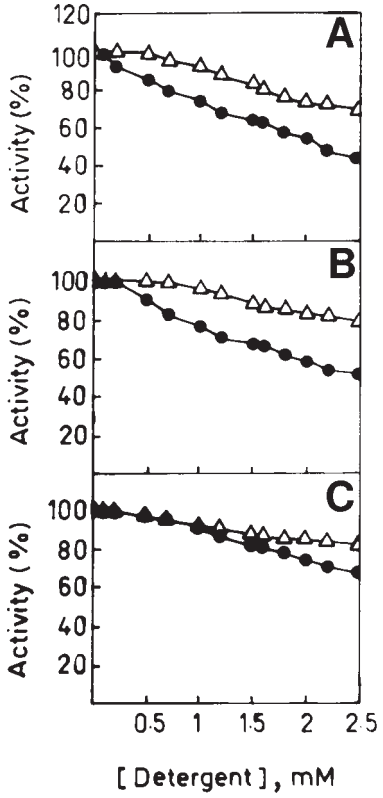
A
B
C
Activity (%)
120
100
80
60
40
20
0.5
1
1.5
2
2.5
[Detergent], mM

Fig. 4

Discussion

In the present study, a comparative study was performed on the effects of extreme conditions such as high temperatures, low and high pH values, organic solvents and chemical denaturing agents on the stability of BLA and BAA. The results indicate substantial differences in the behavior of these enzymes in relation to some of these factors that must be explored in terms of the differences in their primary structures.

A simple scheme describing irreversible thermoinactivation of a protein has been proposed by Klibanov (*see* refs. *8* and *9*). This scheme consists of a reversible denaturation step followed by a covalent irreversible step:

$$N \rightleftharpoons D \longrightarrow I$$

in which *N* is the native form, *D* is the partially unfolded form, and *I* is the totally denatured (inactivated) form.

An important parameter determining the stability of a protein structure is the magnitude of the energy differences (ΔG_D) between the *N* and *D* states *(4,5,10)*. Accordingly, any factor that would lower the energy level of the *N* state or increase the energy level of the *D* state would contribute toward a greater ΔG_D, and thus stabilization of the protein structure *(10)*.

It has been shown that lowering of the energy level of the *N* state (i.e., its stabilization) may be achieved by enhancement of the hydrophobic interactions in the interior or surface of a protein molecule *(10,14)*. Also, in addition to other noncovalent interactions, electrostatic forces may play an important role in stabilization *(23)*. When a protein is placed in an unfavorable environment, certain stabilizing interactions are diminished with the result that the equilibrium is shifted toward formation of a denatured (*D*) state. And, if further unfavorable conditions prevail, the process is completed by the protein being irreversibly inactivated. Of the factors contributing toward the second phase of events, deamidation of asparagine and glutamine has been shown to be particularly important *(8–10)*.

As indicated in Fig. 1, the half-lives of BAA and BLA were 3 and 13 min, respectively, under our experimental conditions. Of the three pH values tested in this connection, the largest differences were at pH 7.0, at which the catalytic potential of the enzymes are normally investigated.

Fig. 3. *(previous page)* Effect of *n*-octane (○) and toluene (△) on thermostability of BLA **(A)** and BAA **(B)** at different temperatures. A 1 mg/mL solution of each enzyme in 20 m*M* Tris, pH 7.5, was aliquoted in 200-µL quantities, which were then lyophilized. Then 200 µL of the solvents or water (control) were separately added and incubated at the required temperature for 3 h. Samples were then evaporated to dryness, and 200 µL of the Tris buffer was added followed by activity determination.

Fig. 4. *(previous page)* Effect of SDS **(A)**, DTAB **(B)**, and SB-12 **(C)** on the catalytic activities of BLA (△) and BAA (●) at 60°C. The detergents were separately added to the assay medium at the indicated concentrations and activities determined in the usual manner.

In comparing the structures of BAA and BLA *(23)*, investigators have suggested that four ionic interactions present in BLA, which cannot be formed in BAA, might be responsible for enhanced thermostability of BLA *(9,23)*.

As indicated in Table 1, all four additives used in this study, without exception, enhanced the thermal stabilities of these enzymes and, at the same time, diminished the extent of deamidation. However, the results presented in Table 2 show significant differences in their effects on T_m. It is clear that while trehalose caused lowering of T_m, the other additives increased it. Based on the proposed mechanisms of stabilization-destabilization outlined above, we suggest that sorbitol, mannitol, and glycerol increase thermal stability by shifting the equilibrium in the reversible step (i.e., increase in T_m) and also by lowering the extent of deamidation. In other words, these additives cause both thermodynamic and kinetic stabilization as defined by Imoto *(10)*. However, trehalose affords protection by preventing deamidation while causing destabilization of the enzymes in the reversible step with the net result being enhanced thermostability. Similar results have been reported on the effect of trifluoroethanol on thermostability of lysozyme *(15)*. This additive was found to enhance irreversible thermostability by lowering T_m while suppressing deamidation *(15)*.

A closer analysis of the results presented in Tables 1 and 2 indicates that sorbitol, which stabilized the two enzymes most effectively, was able to achieve effective stabilization by virtue of its ability to totally prevent deamidation (like trehalose) and, at the same time, increase T_m to the highest extent. The overall thermal stabilization afforded for the mesophilic and thermophilic enzymes may be taken to support earlier reports suggesting polyols as general stabilizers for proteins *(3,14)*.

Deamidation of an asparagine residue proceeds via formation of a five-membered succinimide intermediate that has been suggested to be the most frequent form of deterioration of polypeptides *(24–26)*. This occurs as a result of a nucleophilic attack on the side chain carboxyl carbon of asparagine by the backbone nitrogen of the adjacent amino group *(24–26)*. Accordingly, a higher extent of denaturation observed at low pH values (Fig. 2) is suggested to be the result of a higher degree of protonation of the amino group of asparagine, making it a better leaving group.

It has been reported that thermal stability of many proteins is dramatically increased in certain organic solvents *(6,7)* owing to an increase in the rigidity of the protein structure in these nonaqueous environments *(18–20)*. As shown in Fig. 3, *n*-octane and toluene, which are two immiscible organic solvents with relatively high log *P* values of 4.5 and 2.5, respectively, brought about substantial thermal stabilization. Relative to stability in water, the results are more dramatic for BAA, which behaves like BLA in that total activity is maintained at all temperatures in the experimental conditions utilized. Thus, the nonaqueous environments provided by these organic solvents may induce structural rigidity in the proteins. Furthermore, the differences in thermal stabilities obtained in water, which reflect

their inherently different structural flexibility, is no longer observed in the two organic solvents used in this investigation.

In conclusion, the results presented in this article indicate a higher resistance in the thermophilic enzyme toward denaturation, evidently owing to differences in the structural rigidity of these two protein molecules. Such differences were not detected in nonaqueous environments provided by the organic solvents utilized. Furthermore, sorbitol, which afforded the highest degree of protection, was found to achieve protection by virtue of its ability to increase T_m and, at the same time, to prevent deamidation of these proteins.

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