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Proteolysis of Mesophilic and Thermophilic α-Amylases

A Comparative Study

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Abstract

A comparative study was performed on limited and extensive proteolysis of mesophilic (from *Bacillus amyloliquefaciens* [BAA]) and thermophilic (from *Bacillus licheniformis* [BLA]) α-amylases using trypsin. As expected, the thermophilic enzyme showed greater resistance to digestion by the protease. While the catalytic potential of BLA was enhanced on proteolysis, that of BAA was diminished owing to this process. Combined with greater catalytic activity, a lower thermal stability was observed for BLA on proteolytic treatment. For both enzymes, the extent of proteolytic cleavage was reduced in the presence of various stabilizing agents. The digestion patterns are explained in terms of available information in the literature on the structure of these proteins, especially in relation to segmental mobility.

Index Entries: Mesophilic-thermophilic α-amylases; proteolytic digestion; stabilizers; segmental mobility; apparent molar volume.

Introduction

Numerous studies conducted in the past two decades have established that thermophilic proteins (enzymes) enjoy higher degrees of structural rigidity in comparison with their mesophilic counterparts *(1–4)*. In several such comparative studies *(5,6)*, these proteins showed greater resistance to proteolysis, an observation of utmost importance for both academic and practical reasons. A correlation between sites of proteolysis and segmental mobility in these proteins has also been established *(7–11)*. Furthermore,

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it has been concluded that the sites of cleavage occur at surface loops in the polypeptide chains, and segmental mobility constitutes an essential element of this process *(7)*. It is noteworthy that the variation by residue number of the B-factors shows several mobile or flexible areas, which generally lie in loops and not in helical regions of the protein structure *(7,12–15)*. Since bacterial α-amylases are widely used in industries *(16,17)*, and in the majority of cases at high temperatures, a clear understanding of the structural basis of their rigidity-thermostability is valuable.

In the present investigation, a comparative study was carried out on the resistance of a thermophilic α-amylase obtained from BLA and its mesophilic counterpart extracted from BAA to proteolysis by trypsin. The limited and extensive digestion patterns obtained are explained in terms of structural differences in these proteins based on suggestions in the literature. Part of such comparative studies on the effect of various denaturing agents, organic solvents, and stabilizers has been reported recently *(18)*.

Materials and Methods

Materials

Thermostable α-amylase from *B. licheniformis* was obtained from Novo (Novo Industry A/S, Copenhagen, Denmark). All of the following materials were purchased from Sigma (St. Louis, MO): α-amylase from *B. amyloliquefaciens*, trypsin type IX from porcine pancreas, N_{α}-benzoyl-L-arginine ρ-nitroanilide (L-BAPNA), sodium dodecyl sulfate (SDS), Coomassie brilliant blue R250, Tris, sorbitol, mannitol, and trehalose. Acrylamide, bisacrylamide, TEMED, glycerol, and all other chemicals were obtained from Merck (Darmstadt, Germany). Ammonium persulfate was from Bio-Rad (Richmond, CA) and ρ-nitrophenyl α-D-maltoheptaoside (EPS), was provided by Boehringer Mannheim (Mannheim, Germany).

Densitometry

Densities were measured at 25°C with a precision of $\pm 1.5 \times 10^{-6}$ g/cm^3 using a vibrating tube densimeter (DMA-60, Anton Paar, Austria). The apparent molar volumes, ϕV, of proteins were calculated from the following well-known relationship *(19)*:

$$\phi V = (M/\rho_0) - (\rho - \rho_0)/\rho_0 C)$$

in which M is the molecular weight of protein, C is its molar concentration, and ρ and ρ_o are the densities of the solution and the solvent (buffer), respectively.

Proteolytic cleavage of BAA and BLA

Digestion of BAA and BLA were performed by incubation of the solution of each protein in 10 m*M* Tris and 20 m*M* $CaCl_2$, pH 7.5, with

trypsin (protease:substrate ratio, 1:7) at 37°C for 24 h. At the end of 24 h, required volumes were removed from the reaction mixtures for measurement of the remaining α-amylase activity and for SDS polyacrylamide gel electrophoresis.

Electrophoretic Procedure

SDS gel electrophoresis was performed using 13.5% acrylamide gel according to Laemmli *(20)*. Gels were stained with Coomassie brilliant blue R250 and, when desired, scanned using a Hellena densitometer.

Determination of Enzymatic Activities and Protein Concentration

α-Amylase was assayed at 37°C, using blocked EPS as a substrate at pH 7.5 in 20 m*M* Tris. This procedure is based on the progressive hydrolysis of the substrate, gradually releasing ρ-nitrophenol with an absorption maximum at 405 nm *(21)*.

The activity of trypsin was determined spectrophotometrically using L-BAPNA as substrate. The amount of 4-nitroaniline released per unit time, monitored by the increase in absorbance at 405 nm, was taken as a measure of the catalytic activity of trypsin *(22)*. Protein concentration was determined by the Lowry et al. *(23)* method.

Limited Proteolysis of BAA and BLA

Experiments were also carried out to monitor digestion of BLA and BAA with trypsin at various specific times. Aliquots were removed from the reaction mixtures and phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1 m*M* to inhibit further digestion. Samples were placed in sample solvent immediately.

Modification of Lysine Residues in BAA

Protein modification was carried out using citraconic anhydride as a specific blocking agent for lysine residues and following the procedure described by Dixon and Perham *(24)*. The protein was used at a 3 mg/mL concentration in 10 mL of 100 m*M* borate (pH 8.0), and modification was followed at room temperature by stepwise addition of 3-µL aliquots of the modifier while maintaining the pH of the stirred solution at 8.0 by the addition of 2 *M* NaOH. The reaction was complete on the addition of 15 µL of citraconic anhydride in a total time of 30 min, at which point the pH of the solution remained stable. The reaction mixture was then dialyzed extensively against 20 m*M* Tris, pH 7.5.

Determination of Thermal Stability

The time courses of irreversible thermoinactivation of α-amylase and α-amylase treated with trypsin were measured by incubating the enzyme in 20 m*M* Tris buffer, pH 7.5, at 85°C. 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%).

T_m Measurement

T_m measurement was done using a circular dichroism (CD) spectropolarimeter (JASCO J-715) employing a protein concentration of 0.2 mg/mL. Thermal unfolding was monitored by recording the change of the CD signal at 220 nm as a function of sample temperature. The rate of increase in temperature was adjusted at 1°C/min.

Results and Discussion

Thermophilic proteins are more rigid compared with their mesophilic counterparts *(3,4,25–27)*. This decrease in flexibility, which is the cause of the frequently poor catalytic activity of these enzymes at ambient temperatures, is normally considered as the reason for the preservation of the three-dimensional structure of these proteins over a range of physical and chemical conditions.

BLA, a thermophilic enzyme, consists of a single polypeptide chain of 483 amino acids and catalyzes optimally at about 90°C, a temperature quite different from that required by some of the highly homologous α-amylases from other bacterial sources, such as BAA, a mesophilic enzyme *(25,28,29)*. Because in the majority of industrial applications α-amylases are used at high temperatures, much effort has been directed toward elucidating the structural basis of thermostability in these enzymes *(30–34)*.

In the present investigation, BLA was compared with BAA in relation to proteolysis by trypsin, and the nature of the site of attack (Lys/Arg) was deciphered using a specific modifier of lysine residues. The results are explained in terms of structural differences in these proteins utilizing information provided in the literature.

Proteolysis and Effect of Modification

Proteolysis was performed by incubating BAA and BLA with trypsin for 24 h. As indicated in Fig. 1 and Table 1, digestion was more extensive for BAA compared with BLA. For example, more than twice the amount of BLA (56 kDa) resisted proteolysis compared with BAA. Also, three bands (44, 21, and 9 kDa), which were almost undetectable in BLA, were clearly observed in BAA.

Limited proteolysis was also carried out for the two enzymes (Fig. 2A,B). The initial sites of proteolysis were indicated by the appearance of a 47- and a 38-kDa band on the gels, corresponding to limited tryptic digests of BAA and BLA, respectively. A very faint 44-kDa band that appears on limited digestion of BAA (and not in BLA) clearly appears on extensive digestion of the enzyme (Fig. 1, Table 1).

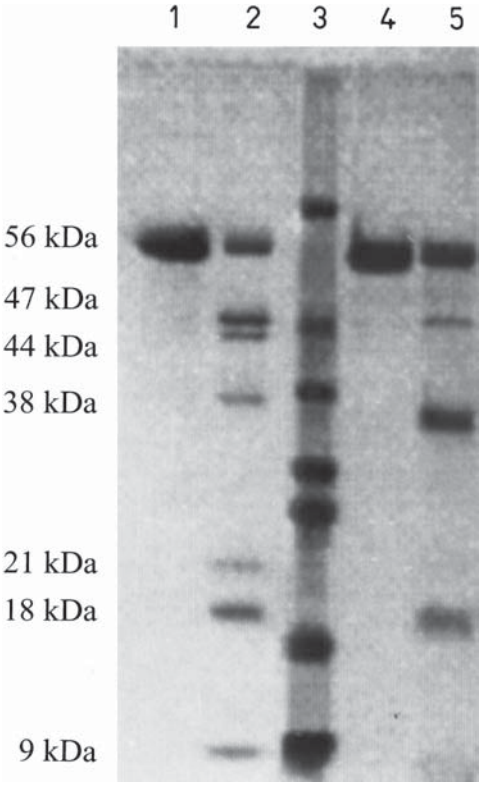

Fig. 1. Extensive proteolysis of BAA (lane 2) and BLA (lane 5) by trypsin. Fifty microliters of the samples was applied in each lane. Before mixing with 2X Laemmli, the samples contained 0.7 mg/mL of BAA or BLA. Digestion was carried out for 24 h at 37°C in the presence of 0.1 mg/mL of trypsin. Lanes 1 and 4 are controls for BAA and BLA, respectively, and lane 3 represents the bands corresponding to standards. On the left, the molecular weights of the fragments corresponding to digestion of BAA and BLA, respectively, are indicated.

Table 1
Extensive Proteolysis of BAA and BLA[a]

Molecular weight (kDa)	BAA (%)	BLA (%)
56	20	44
47	17	12
44	14	0
38	11	28
21	9	1
18	17	15
9	12	0

[a]Molecular weights of the observed bands together with representative percentages are indicated. For details, see the legend to Fig. 1 and Materials and Methods.

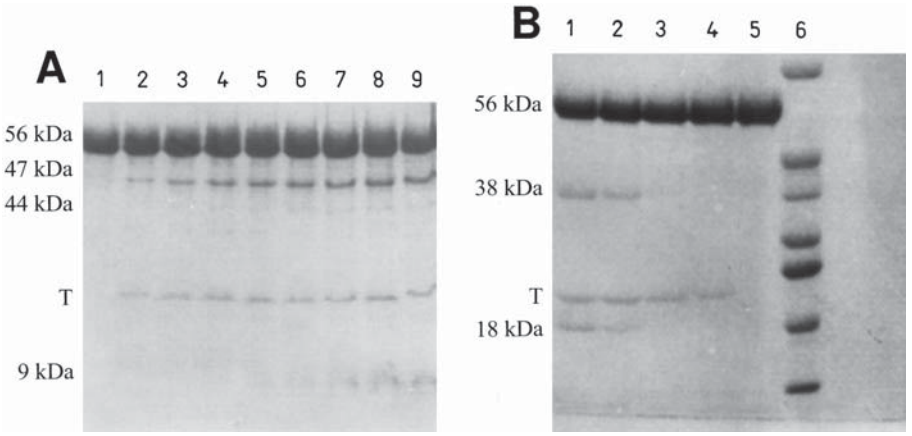

Fig. 2. Limited proteolysis of BAA **(A)** and BLA **(B)** with trypsin (T). Details are exactly as described in the legend to Fig. 1. (A) lane 1, control; lane 2, sample taken immediately after mixing; and lanes 3–9, 5, 10, 15, 20, 25, 30, and 45 min of digestion, respectively. For (B), lane 6, standard; lane 5, control; lane 4, sample taken immediately after mixing; lanes 1–3, 60, 25, and 5 min of digestion, respectively.

Several mobile-flexible areas that generally lie in the loop regions of the protein structure were proposed as potential candidates for sites of proteolysis in BLA *(15)*. Because no such structural information was available for BAA, an attempt was made in this direction by carrying out modification studies. As shown in Fig. 3, when BAA was chemically modified by citraconic anhydride, used in this investigation as a specific blocking agent of lysine residue, the 47-kDa band presented in Fig. 2A no longer appeared, thus indicating that the site of attack at this point consists of a lysine and not an arginine residue.

Limited proteolysis was found to bring about changes in the catalytic efficiencies of the two enzymes (Fig. 4). Note that in the case of the thermophilic enzyme, activation is obtained, presumably owing to induction of flexibility in its relatively rigid structure *(4,35,36)*. However, the mesophilic protein, which is inherently a flexible structure, lost some of its catalytic potential on proteolysis. In this regard, it has been shown that the thermostability of the enzyme was clearly decreased by proteolytic treatment (Fig. 5). Also, by CD measurements, it was found that the T_m of the native enzyme was diminished from 89 to 86°C, a further indication of enhanced flexibility owing to this process.

The relationship between structural flexibility and catalytic activity indicated in the present study has been reported by other investigators. Thus, it has been shown that while a thermophilic 3-isopropylmalate dehydrogenase is relatively more rigid and less active than its mesophilic counterpart at room temperature, the two enzymes exhibited very similar flexibility at temperatures near their activity optima *(37)*. In addition,

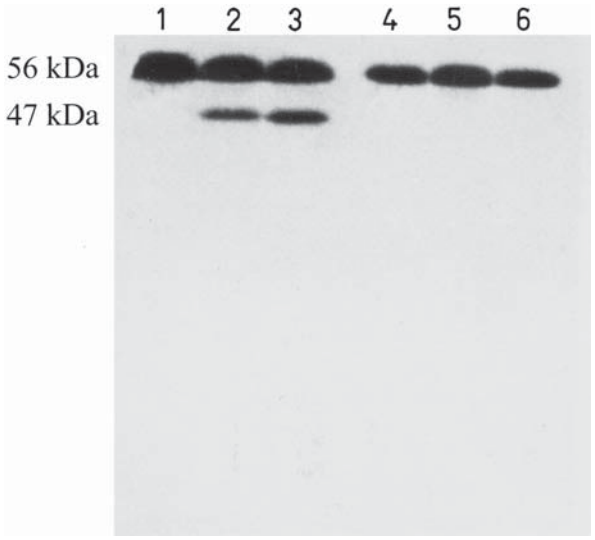

Fig. 3. Effect of modification of BAA by citraconic anhydride on its limited digestion by trypsin. To 10 mL of a 3 mg/mL solution of BAA, 15 µL of the modifier was gradually added in a total time of 30 min at room temperature. Other details are as described in Materials and Methods. Lanes 1–3, unmodified BAA; lanes 4–6, modified BAA; lane 1 (4), control; lanes 2 (5) and 3 (6), after 10 and 45 min of digestion, respectively.

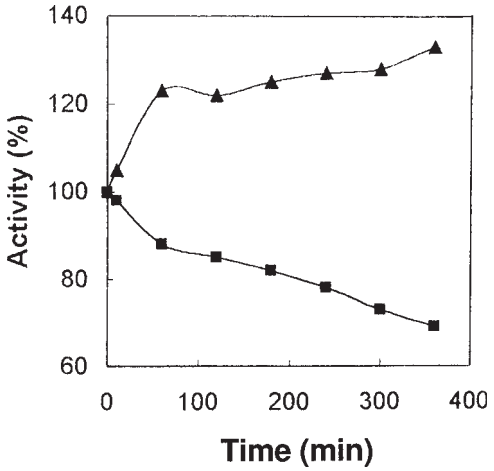

Fig. 4. Effect of limited proteolysis on catalytic efficiencies of the α-amylases. Reaction mixtures containing BAA (■) and BLA (▲) at 0.7 mg/mL concentration in the presence of 0.1 mg/mL of trypsin were incubated at 37°C, and at various times samples were withdrawn and activities determined in the usual manner. For further details, see Materials and Methods.

several reports in the field of nonaqueous enzymology *(38,39)* have clearly suggested conformational rigidity as a major factor leading to diminished catalytic activity.

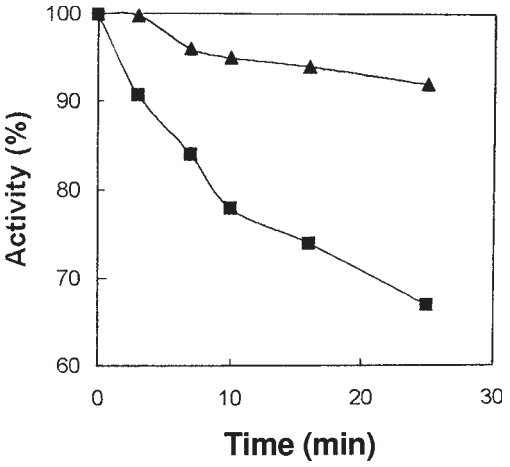

Fig. 5. Effect of limited proteolysis of BLA on its thermal stability. Digestion was carried out for 90 min, and proteolysis was terminated by the addition of PMSF at a final concentration of 1 m*M*. The reaction mixture was then incubated at 85°C, and at the times indicated, samples were removed and cooled on ice and activities determined in the usual manner. For the control, exactly the same procedure was followed with the only exception that trypsin was not included. Data for control (▲) and BLA treated with trypsin (■) are indicated.

A clear correlation between segmental mobility and sites of limited proteolysis in globular proteins has been established *(7–11)*. It has further been concluded that sites of polypeptide chains characterized by highest mobility, normally occurring in exposed loops, are relatively more susceptible to proteolytic cleavage. Based on the three-dimensional structure of a calcium-free form of BLA, five of such flexible areas have been identified *(15)*. These sites include regions 120–133, 370–376, 413–422, 440–448, and 458–467 *(15)*. Cleavage at the first four sites, which contain lysine and/or arginine residues, would result in peptides with approximate masses of 16,39; 43,12; 47,8; and 50,5 kDa, respectively.

From the primary structure of BLA *(15,40)*, it is evident that the first region (120–133) consists of two arginine residues and no lysine—the highest number of arginine residues in the five flexible regions described. This, together with the fact that in digestion by trypsin there is a 2- to 10-fold preference for arginine over lysine *(41,42)*, explains why this region is first cleaved by trypsin. Cleavage at these sites results in the appearance of two major fragments with molecular masses of 18 and 38 kDa, as indicated in Fig. 2B. On the other hand, this region is not the primary target of attack in BAA.

In connection with the data just discussed, the following points are noteworthy and of special relevance. One of the two arginine residues in BLA (Arg 127) of the first flexible region (120–133) has been changed into glutamine in BAA *(15,40)*. On longer digestion of BAA, the two bands corresponding to molecular masses of 18 and 38 kDa do appear (Fig. 1).

As already discussed, the results obtained from chemical modification of BAA, using citraconic anhydride as a specific modifier of lysine residues, indicated that a site consisting of lysine and not arginine is the primary target for digestion by trypsin. In the limited digestion pattern of BAA, a 47-kDa band appears, which suggests that the third flexible region described for BLA (sequence 413–422) is actually the site primarily cleaved (Fig. 2A). In this region in BAA *(15,40)*, there is one arginine (also present in BLA) and a lysine (asparagine in BLA). Also note that in the primary structure of α-amylase from *Bacillus stearothermophilus*, this is the least conserved region with six changes in a row expected to constitute a highly accessible surface region in the protein structure *(43,44)*.

Another point warranting discussion is the difference in the extensive digestion patterns observed for BAA and BLA. As indicated in Fig. 1, a 44-kDa band that is barely observable as a result of limited digestion of BAA is clearly formed on prolonged digestion of this enzyme and not of BLA. As pointed out, region 370–376 in BLA constitutes one of the predicted flexible sites *(15)*. In this region, there are one lysine (370) residue and one arginine (375) residue. Of these, Arg 375 has been shown to interact ionically with Glu 336 *(15)*, an unfavorable condition related to cleavage at this site. On the other hand, at this position in BAA, there is a lysine instead of an arginine. Furthermore, at the P2 position of this lysine there is a proline. The existence of this proline has been shown to help align this site for productive binding to the protease *(45)*.

Although the five flexible regions referred to have been suggested based on X-ray diffraction results for a Ca^{++}-free BLA, it nevertheless appears that the nature of the proteolytic pattern obtained in the present study (with Ca^{++} included) is best explained using these deductions *(15)*. Thus, the facts that conformational changes in BLA may take place on binding or removal of Ca^{++} *(15)* and that the thermostability of this protein is enhanced by the presence of this divalent ion are not considered in the present discussion. Also note that the flexible region in BAA proposed by Suzuki et al. *(34)* appears to remain unaffected by the limited proteolytic treatment of the enzyme described in the present investigation.

Effect of Additives

A number of additives that have been shown to enhance thermostability of BAA and BLA *(18,46)* were tested in connection with proteolysis. As shown in Fig. 6, a higher degree of catalytic potential of BAA remained on treatment with trypsin when these additives were included in the digestion media. This corresponded to a lower degree of proteolysis, as indicated in Fig. 7 and Table 2 (in which results obtained using trehalose are presented as a typical effect). Regarding BLA, the activation presented in Fig. 3 was also observed in the presence of the additives (results not shown). The extent of activation was almost the same as for control, irrespective of the type of additive used. These results are in accordance with

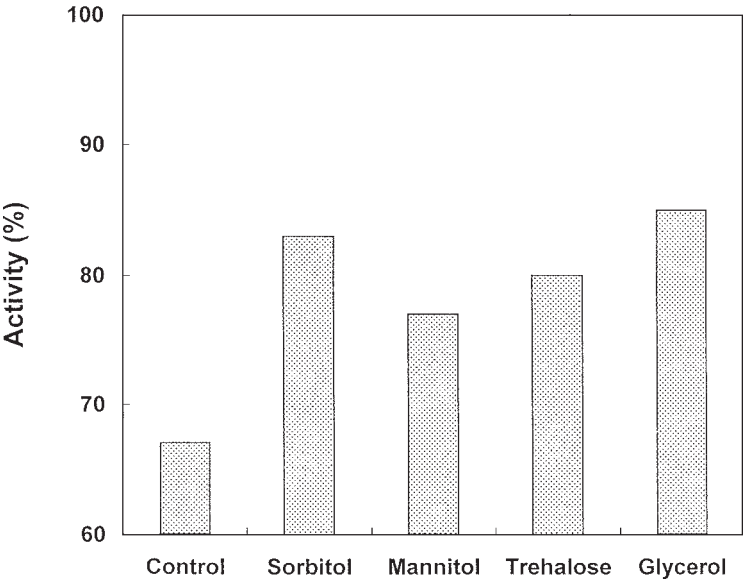

Fig. 6. Effect of various additives on proteolysis of BAA. Digestion with trypsin was carried out in the usual manner for 6 h. At this point, samples were withdrawn and activities determined. For further details, see Materials and Methods.

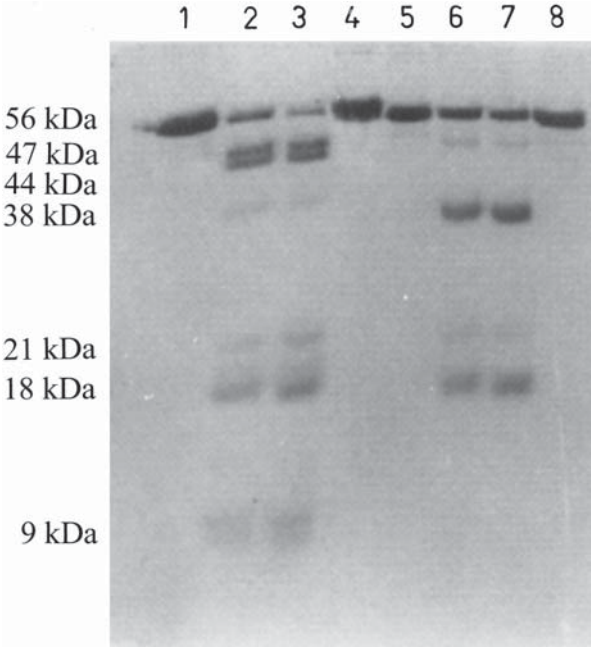

Fig. 7. Effect of trehalose on proteolytic digestion of BAA and BLA. Digestion with trypsin was carried out in the absence and presence of trehalose (20% [w/v]) for 6 h. Further details are as described in the legend to Fig. 1 and the text. Lanes 1–4 are for BAA and lanes 5–8 are for BLA; lane 1 (5), control in the presence of trehalose; lane 4 (8), control in the absence of trehalose; lanes 3 (7) and 2 (6), digestion patterns in the absence and presence of trehalose, respectively.

Table 2
Effect of Trehalose on Digestion of BAA and BLA by Trypsin[a]

Molecular weight (kDa)	BAA (BLA) (–trehalose)	BAA (BLA) (+trehalose)
56	19 (39)	29 (49)
47	15 (6)	17 (7)
44	12 (0)	14 (0)
38	5 (31)	6 (25)
21	13 (3)	9 (2)
18	21 (21)	15 (17)
9	15 (0)	10 (0)

[a]Molecular weights of the observed bands together with representative percentages in the absence and presence of trehalose are indicated for the two enzymes. Numbers in parentheses are for BLA. For details, see the legend to Fig. 7 and Materials and Methods.

our previous findings on the effectiveness of these additives at diminishing the extent of irreversible thermoinactivation *(18)*.

A lower degree of proteolysis obtained in the presence of the aforementioned additives is suggested to occur as a result of the stabilization (or "rigidifying") effect of these cosolvents. This is presumably an outcome of preferential hydration of the proteins *(47–49)* owing to the effect of the additives on the surface-free energy of water *(50)*.

Catalytic activity of trypsin by itself (control) was determined when the protease was incubated at 37°C in the absence and presence of various additives for 6 h. No difference in its catalytic potential could be detected in all cases examined, using L-BAPNA as substrate.

Apparent Molar Volume

Additional support for the differences in the degree of compactness between the mesophilic and thermophilic proteins used in this study was sought by determining their apparent molar volumes (ϕV). This was done by measuring density following the procedure described in Materials and Methods. Values of 18,630 and 14,695 cm^3/mol, respectively, for BAA and BLA indicated that indeed the thermophilic protein is relatively more compact in comparison with its mesophilic counterpart. Relatively high packing densities for hyperthermophilic proteins have already been reported *(25,51)*.

Conclusion

The results presented in this comparative study indicate a higher resistance of the thermophilic enzyme to digestion by trypsin, apparently owing to a greater degree of structural rigidity of this protein. Proteolytic treatment of the enzyme brought about enhancement of its catalytic poten-

tial evidently owing to increased conformational mobility of this otherwise rigid and compact molecule. The use of citraconic anhydride as a specific modifier of lysine residues provided information on the nature of the site of cleavage. The limited digestion patterns obtained are in accordance with suggestions in the literature on positioning of flexible areas (loops) in the protein structures that constitute the primary targets for proteolytic attacks. Several additives have been shown to diminish proteolysis similar to their protection against other unfavorable conditions described recently *(18)*.

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