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THERMOSTABILITY AT ULTRAHIGH TEMPERATURES OF THERMOLYSIN AND A PROTEASE FROM A PSYCHROTROPHIC *PSEUDOMONAS* *

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Summary

Thermal inactivation at 110—150°C of thermolysin (EC 3.4.24.4), produced by the thermophile *Bacillus thermoproteolyticus*, and the extracellular protease of *Pseudomonas* sp. MC60 a psychrotroph, were investigated at 130°C, both enzymes had approximately the same $\Delta H^{\star}$ (22 kcal/mol) and $\Delta S^{\star}$ (—13.5 cal/mol per degree) values. Both enzymes contain zinc and calcium. The amino acid compositions of the enzymes were similar except that MC60 protease exhibited a more typical tyrosine content. Comparable heat resistance at extreme temperatures of enzyme produced by psychrotrophic and thermophilic organisms emphasizes the difference between molecular properties that resist denaturation at elevated temperatures and those that allow reversible denaturation.

Introduction

The mechanism of thermostability of proteins from thermophilic microorganisms is still unknown. Classically, enzyme heat stability has been attributed to the stabilizing effect of calcium ions, and the role of calcium in the thermostability of thermolysin has been extensively investigated [1—5]. In another study of thermolysin, however, it was reported there was nothing particularly unusual about the protein's conformation which could explain its high thermostability [6]. In fact, a comparison of many enzymes from thermophiles with their mesophilic counterparts led to the conclusion that, except for some minor differences, proteins from both classes of microorganisms appear to be physicochemically similar [7]. Comparative studies of heat-resistant and heat-labile

* Thermolysin (protease from *Bacillus thermoproteolyticus* Rokko). Paper No. 5166 of the Journal Series of the North Carolina Agriculatural Experiment Station, Raleigh, North Carolina, U.S.A.

enzymes may be necessary to ultimately define the factors responsible for differences in thermal stability [8].

Previous studies of thermolysin (EC 3.4.24.4) and other proteolytic enzymes have used the conformational changes that lead to autolysis as an index of thermal resistance; however, such a parameter more closely measures localized denaturation and not necessarily thermal inactivation. Current authors have concluded that it is difficult to ascertain the heat-resistance and mechanism of thermal stabilization of these enzymes under conditions where autolysis occurs [3,9].

In this investigation, we examined the kinetics of thermal inactivation of an extracellular heat-stable protease produced by a psychrotrophic bacterium and thermolysin, a classical heat-resistant protease from a thermophilic organism, at temperatures (110—150°C) which should obviate interfering effects of autolysis. We were interested in the ability of these enzymes to survive thermal treatment rather than their ability to maintain activity at elevated temperatures and in some of the characteristcs of these enzymes which may contribute to this thermal stability.

Materials and Methods

Materials. Thermolysin, crystallized and lyophilized, was purchased from Sigma Chemical Company (St. Louis, Mo., U.S.A.). Protease from *Pseudomonas* sp. MC60 was obtained and purified as described previously [9]. Purity was confirmed by sodium dodecyl sulfate (SDS) gel electrophoresis. Vitamin-free casein was obtained from Nutritional Biochemicals Corporation (Cleveland, Ohio).

Heat treatments. Thermolysin solutions were prepared by adding the crystalline enzyme to 0.5 M Tris · HCl buffer, pH 7.2, containing 0.1 M $CaCl_2$, MC60 protease solutions were made with 0.1 M Tris · HCl buffer and 0.01 M $CaCl_2$, pH 7.0. Each buffered enzyme solution was heated in sealed glass capillary tubes in a temperature-controlled oil bath. Heated tubes were cooled rapidly in an ice/water mixture, and the contents were added to the casein substrate. The come-up time of the fluid in the tube was approximately 10 s, as measured by a thermocouple. If samples were heated for 60 s or less, correction for heat penetration lag was made.

Enzyme assay. Proteolytic activity was measured spectrophotometrically by the method of Hull using the Folin-Ciocalteau reagent [10]. The substrate solution was 3.5% vitamin-free casein in 5 mM $CaCl_2$/0.5 mM $ZnSO_4$ (pH 7.5). Enzyme activity was assayed at 45°C and the reaction was terminated by the addition of 0.72 M trichloroacetic acid. Substrate concentration was in saturating amounts, and reaction rates were linear with the concentrations of protease used.

Molecular weight estimation. The molecular weight of MC60 protease was estimated by gel electrophoresis in the presence of 0.1% SDS [11]. Protease was treated with 0.1 M EDTA, pH 7.0 for 30 min at 25°C before samples were applied to the 10% acrylamide gel. Failure to remove metal ions from the protease resulted in autolysis during electrophoresis as indicated by the appearance

of several bands of lower molecular weights than that observed for the EDTA-treated protein.

Metal ion determinations. Calcium and zinc were determined using a Jarrell-Ash (Model 810) atomic absorption spectrophotometer with a 10 cm premix laminar flow burner. Absorbance was measured at 422.2 nm (calcium) and 213.9 nm (zinc) with 7 lb/inch^2 of acetylene and 40 lb/inch^2 of supporting air and with 8 mA applied to each hollow cathode lamp. Standard curves were obtained for a range of 25—1000 ppb for each metal.

Amino acid analysis. MC60 protease was dried and weighed in Pyrex vials. Protein samples were acid hydrolyzed (6 M HCl) in evacuated vials placed in a refluxing toluene bath for 24 h. Amino acid analyses were performed with a Beckman model 116 automatic amino acid analyzer. Amino acid composition was calculated on the basis of recovery compared to a standard solution containing 0.1 μmol of each amino acid and ammonia. Tryptophan was estimated according to the method of Spies and Chambers [12]. The content of cysteine was estimated according to the method of Ellman [13] after treatment of the protein with 8.5 M urea.

Results and Discussion

Thermolysin heated at ultrahigh temperatures in the presence of calcium did not follow tryptical inactivation kinetics. We observed biphasic inactivation curves on a plot of log percent activity vs. heating time which suggested both autolysis and thermal inactivation had occurred. Increased autolysis in addition to thermal inactivation at elevated temperatures was first recognized by Feder, Garrett and Wildi [1]. The first phase of inactivation was very rapid and represented a loss of activity varying between approx. 90% at 110°C to 30% at 150°C. As indicated here and by others, autolysis of thermolysin during heating was a major contributor to activity loss [3]. The linear second phases were assumed to represent first-order inactivation kinetics and rate constants (k) were determined using these slopes. Linear thermal inactivation curves for MC60 protease indicated no autolysis of this enzyme occurred at ultrahigh temperatures. This was probably due to the lower optimum temperature for MC60 protease activity [14]. We observed the same denaturation kinetics for thermolysin at lower temperatures (80—95°C) as had been reported elsewhere [4].

Arrhenius plots were used to compare the ultrahigh temperature inactivation of thermolysin and MC60 protease (Fig. 1). Clearly, there was little difference in the thermostability of these enzymes at temperatures of 110—150°C. Comparable rates of inactivation over the ultrahigh temperature range suggested that the mechanisms of inactivation were similar. The slope change observed for both enzymes indicates an altered protein heat-capacity (C_p) which allowed an accelerated thermal inactivation at temperatures above 130°C. The values for activation enthalpy (ΔH^*), entropy (ΔS^*), and standard free-energy (ΔF^*) calculated from transition state theory, were similar for the two enzymes (Table I). The ΔH^* values we obtained for both thermolysin and MC60 protease at ultrahigh temperatures were 0.5—0.25 of those typically observed for enzyme denaturation. Our activation parameters for thermolysin were lower than an average ΔH^* of 76 kcal/mol and an average ΔS^* of +137 cal/mol per degree

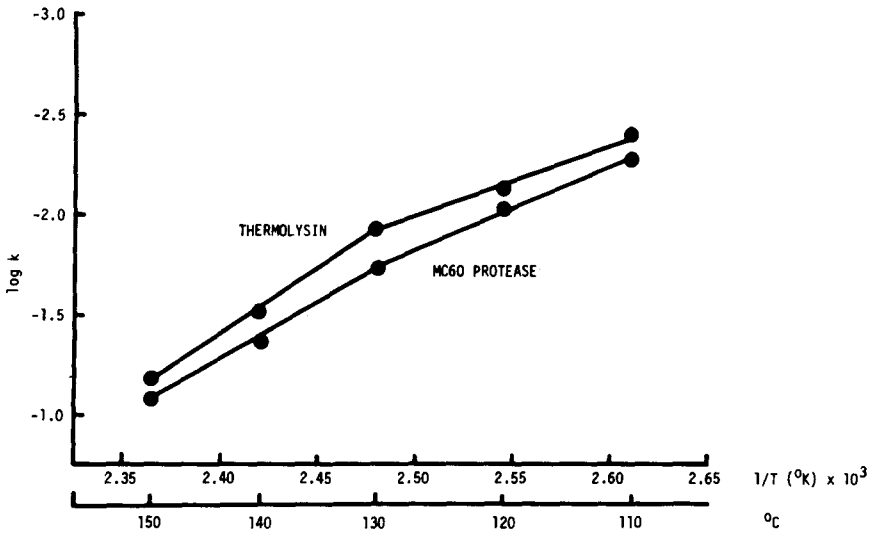

Fig. 1. Arrhenius plot of the heat inactivation of thermolysin and *Pseudomonas* sp. MC60 protease at ultrahigh temperatures.

calculated from literature values [4,5,15]. These values, however, were based on experiments in which the enzyme was heated at temperatures where denaturation and subsequent autolysis would occur. Thus, the thermostability we have observed for thermolysin was greater than had been previously expected since the effects of autolysis were eliminated in our calculations. We conclude that the localized denaturation which leads to autolysis has a higher temperature coefficient than the process of inactivation at ultrahigh temperatures. This suggests that all molecules which might become inactivated were first denatured, and that molecules that were not inactivated or autolyzed during the

TABLE I

ACTIVATION PARAMETERS FOR HEAT INACTIVATION OF *PSEUDOMONAS* SP. MC60 PROTEASE AND THERMOLYSIN

Enzyme	Temperature (°C)	ΔH^* (kcal/mol)	ΔS^* (cal/mol per degree)	ΔF^* (kcal/mol)
MC60 Protease	110	18.4	—21.2	26.5
	120	18.4	—21.4	26.8
	130 *	21.5	—13.6	26.9
	140	24.6	—5.7	26.9
	150	24.5	—5.9	27.0
Thermolysin	110	15.5	—29.3	26.7
	120	15.5	—29.2	26.9
	130 *	21.9	—13.4	27.3
	140	28.4	+2.8	27.2
	150	28.3	+2.6	27.3

* Thermodynamic values were based on a slope calculated by regression analysis of points at all temperatures. The linear correction (r) for MC60 protease data was $r = -0.995$ and for thermolysin data was $r = -0.990$.

treatment underwent reversible denaturation. The disruption of hydrophobic interactions and exposure of nonpolar groups to solvent during inactivation were indicated by the low entropy values. Since ΔH^* became more positive with increasing temperature, a positive $\Delta C_p{}^*$ is suggested.

Several similarities exist in the molecular makeup of thermolysin and MC60 protease which may partially account for their unique heat-resistance. Okta, Ogura and Wada [16] found thermolysin to have a molecular weight of 37 500. MC60 protease also was moderately large with a molecular weight estimated as 48 000 by gel filtration [9] and, in this study, 49 000 by SDS gel electrophoresis of the apoenzyme. Amino acid analysis of thermolysin [16] indicated an abundance of amino acids with hydrophobic side chains and the absence of cystine. Like thermolysin, MC60 protease has no measureable cystine and cys-cysteine (Table II).

The lack of cystine in both these enzymes is inconsistent with the general suggestion of Anfinsen [17] that most extracellular proteins, which could require greater stability to environmental changes, usually contain disulfide bond cross-links. Other heat-resistant enzymes also do not contain this type of bond [7,18]. Disulfide bonds have been shown to limit the expansion of proteins under denaturing conditions [19]. Possibly the lack of disulfide cross-links gives these enzymes an added degree of flexibility which allows them to survive the stress of the heat treatment.

Thermostability of thermolysin has been attributed to extensive hydrophobic interactions within the enzyme which were partially formed by the aroma-

TABLE II

AMINO ACID COMPOSITION OF *PSEUDOMONAS* SP. MC60 PROTEASE

The number of residues is taken per 48 500 daltons.

Amino acid			Number of residues per molecule	
	(mmol/100 g protein)	(g/100 g protein)	Calculated	Nearest integral
Aspartic acid	169.4	19.49	82.1	82
Threonine	102.1	10.32	49.5	50
Serine	73.7	6.42	35.8	36
Glutamic acid	57.8	7.46	28.0	28
Proline	15.8	1.53	7.6	8
Glycine	134.7	7.67	65.1	65
Alanine	99.9	7.11	48.4	48
Half-cystine	0	0	0	0
Valine	43.2	4.28	20.9	21
Methionine	4.1	0.54	1.9	2
Isoleucine	38.4	9.25	39.6	40
Leucine	81.7	4.34	18.6	19
Tyrosine	43.0	7.02	20.9	21
Phenylalanine	43.8	6.45	21.3	21
Lysine	25.9	3.32	12.5	13
Histidine	10.4	1.43	5.1	5
Arginine	6.9	1.08	3.4	3
Tryptophan	8.8	0.97	4.3	4
Amide-NH_3	77.6	(1.32)	37.6	(38)
Total		98.68		466

TABLE III

CALCIUM AND ZINC CONTENT OF NATIVE AND EDTA-TREATED *PSEUDOMONAS* SP. MC60 PROTEASE

	Ca^{2+} (μg/g enzyme)	Zn^{2+} (μg/g enzyme)	Ca^{2+} (gatom/48 500 g)	Zn^{2+} (gatom/48 500 g)
Native *	3500	510	4.2	0.4
EDTA-treated **	360	0	0.4	0.0

* Enzyme was dialyzed at 4°C against metal-free 0.1 M Tris · HCl buffer, pH 7.0.
** Enzyme was treated with 0.1 M EDTA, pH 7.0 for 30 min at 25°C and dialyzed.

tic region of abnormally ionizing tyrosines [15]. Other workers suggested that subtle differences in hydrophobic character, metal binding, hydrogen bonding, ionic interactions, or a combination of these were responsible for thermostability [6]. By amino acid analysis, MC60 protease was shown not to have a particularly high tyrosine content, but it was unusually rich in threonine. It had approximately the same percentage of residues with hydrophobic side chains as thermolysin. The normal tyrosine content of MC60 protease indicated an abundance of tyrosine residues was not a prerequisite for thermostability at ultrahigh temperatures. However, the 35-degree difference in otimum temperature for activity of these enzymes, which obviously is dependent on maintenance of enzyme conformation, might be achieved by the hydrophobic interactions of additional tyrosines [15] or by different contributions from calcium ion binding [5] or by a combination of these. Table III shows the metal content of MC60 protease. It indicates that like thermolysin [1], MC60 protease also contains zinc and several calciums. The metal content was influenced by treatment with EDTA. Since bound calcium ions appear to dissociate as the protein "melts" above the temperature optimum [3], these authors speculate that at least one and possibly several calcium ion salt-bridges play no role in maintaining conformation at ultrahigh temperatures. The ability of these metalloenzymes to survive such extreme temperatures most likely reflects structural flexibility and the interplay of divalent cations in allowing rapid and accurate enzyme renaturation rather than maintenance of native structure during heating.

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