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Irreversible Thermoinactivation Mechanisms of Subtilisin Carlsberg

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Received October 30, 1989

In order to find the rational methods for improving the thermal stability of subtilisin Carlsberg, the mechanisms of irreversible thermoinactivation of the enzyme were studied at 90 °C. At pH 4, the main process was hydrolysis of peptide bond. This process followed first order kinetics, yielding a rate constant of $1.26 \times 10^{-1} h^{-1}$. Hydrolysis of peptide bond of PMS-subtilisin occurred at various sites, which produced new distinct fragements of molecular weights of 27.2 KD, 25.9 KD, 25.0 KD, 22.3 KD, 19.0 KD, 17.6 KD, 16.5 KD, 15.7 KD, 15.0 KD, 13.7 KD, and 12.7 KD. Most of the new fragments originated from the acidic hydrolysis at the C-side of aspartic acid residues. However 25.0 KD, 15.7 KD, and 13.7 KD which could not be removed in purification steps stemmed from the autolytic cleavage of subtilisin. The minor process at pH 4 was deamidation at asparagine and/or glutamine residues and some extend of aggregation was also observed. However, the aggregation was main process at pH 7 with a first order kinetic constant of 16 h^{-1}. At pH 9, the main process seemed to be combination of deamidation and cleavage of peptide bond.

Introduction

Inactivation of enzymes by high temperature can be classified into two types of mechanisms. One is the reversible thermoinactivations of enzymes caused by temperature-induced conformational changes in protein structures.[1] These phenomena have been extensively studied with various proteins and their mechanisms are relatively well understood.[2] On the other hand, irreversible thermoinactivations of enzymes caused by a combinations of aggregations, incorrect structure formations, or destruction of primary structure have remained somewhat mysterious, primarily because of severe conceptual and experimental problems encounted in their investigations.[3-5] However, recently, Klibanov group have partly succeeded in elucidating the detailed mechanisms of irreversible thermoinactivation of some well known enzyme such as lysozyme[6] and ribonuclease.[7] The processes leading to thermoinactivations were found to be deamination of Asn/Glu residues, hydrolysis of peptide bond at Asp residues, destruction of disulfide bonds, and formations of incorrectly folded and kinetically trapped structures. These findings provide the basis of rational reasonings for improving thermostabilities of enzymes.[8,9]

In the present work, we examined the thermoinactivation mechanisms of subtilisin Carlsberg in order to search for strategies to improve thermostability of the enzyme. Subtilisin is an extracellular serine protease (also known as alkaline endopeptidase) produced by *Bacillus subtilis*. Today this enzyme is one of the most important microbial protease economically because it is the predominant enzyme used in detergents. In contrast to other members of the serine protease family such as chymotrypsin and trypsin, subtilisin differs from them completely in sequence and tertiary structure but have an essentially identical arrangement of amino acid residues at the active site for its function.[10] This enzyme consists of a single polypeptide chain with 274 amino acids, corresponding to molecular weight of 27,277 daltons.[11] The crystal structures have been determined to 2.5 Å resolution[12]

and more recently at 1.8 Å.[13]

In order to elucidate the mechanisms of irreversible thermoinactivation for subtilisin Carlsberg, the PMSF treated enzyme were heated at 90 °C and at various pHs. The heated samples were then analyzed by several techniques including SDS-PAGE, measurement of turbidity, and determination of ammonia. The results obtained from the present analyses have been compared to those of Klibanov's.[6,7]

Experimental

Materials. Subtilisin Carlsberg (EC 3.4.4.16), L-glutamic dehydrogenase, β-NADH, α-ketoglutarate, phenylisothiocyanate, N-benzoylarginine ethyl ester (BAEE), phenylmethylsulfonylfluoride (PMSF), marker proteins, acrylamide, and bisacrylamide were purchased from Sigma Chemical Co.. Pyridine and *n*-butylacetate were from Jusei Chemical Co. and TLC plastic sheets of silica gel 60F 254 was the product of Merck. All other chemicals used were reagent grade commercially available.

Assay of Subtilisin. Subtilisin activity was measured by differential spectral assay of Hummel with some modifications.[14] An aliquote of subtilisin solution (final 0.1-1 μM) was added to a test tube containing 2.0 m*l* of 0.2 M Tris-HCl buffer (pH 8.5) and 0.6 m*l* of 10 mM BAEE. Then the mixture was transferred immediately to a UV cell of 0.2 cm path length and the hydrolysis of BAEE was measured at 255 nm by a Beckman DV 5270 spectrophotometer. The initial rate of absorbance change was estimated by drawing tangent to the curve obtained during first 30-60 seconds of measurements. One unit of activity is defined as the hydrolysis of one micromole of BAEE per minute at 25 ± 1 °C.

Preparation of PMS-subtilisin Carlsberg. In order to avoid autolysis, the enzyme was inhibited by PMSF which reacts with active site serine-221. Thirty milligrams of subtilisin dissolved in 5 m*l* of 0.1 M phosphate buffer (pH 7.4) was mixed with 0.1 m*l* of an 20 mg/m*l* solution of PMSF in acetone. The solution was stirred for 1 hour at room temperature and the treatment was repeated. After 1 hour, the insoluble particles were removed by filtration through a Millipore filter. The PMS-subtilisin solution was then applied to a Sephadex G-50 column (2.9 × 75 cm) equilibrated with 0.1 M sodium acetate buffer (pH 4), and eluted with the same buffer at an elution rate of 14 m*l*/hr. The fractions containing PMS-subtilisin were dialyzed exhaustively against water and then lyophilized.

Heat Treatment. The lyophilized enzyme was redissolved in an appropriate buffer solution, namely, 0.1 M sodium acetate buffer (pH 4), 0.2 M sodium phosphate buffer (pH 7), or 0.2 M sodium carbonate buffer (pH 9.12). The final concentration of the enzyme was adjusted to 0.25 ± 0.01 mg/m*l* or 1.0 ± 0.10 mg/m*l* depending on the experimental conditions. Microcentrifuge tubes or ampules containing the enzyme solution were then placed in a water bath maintained at 90 °C and the samples were periodically removed. All of the samples were then centrifuged at 10,000 × g for 5 min and the supernatants were analyzed by SDS-PAGE.

SDS-polyacrylamide Gel Electrophoresis. SDS-PAGE was performed using 12.5% acrylamide gel (15 cm × 13 cm × 1.5 mm) by method of Laemmli.[15] The supernatant samples were treated with TCA (final 10%) to isolate proteins and centrifuged at 10,000 × g for 5 min. The precipitated proteins were washed with ethanol-ether (1:1 v/v) to remove TCA and residual ethanol-ether was evaporated under vacuum. The pellets were dissolved directly in sample buffer for SDS-PAGE and then subjected to electrophoresis. The gel was stained with Coomassie brilliant blue and destained with water:methanol:acethic acid (60:30:10, v/v). The protein bands were quantitatively analyzed by DMU-33C digital densitometer.

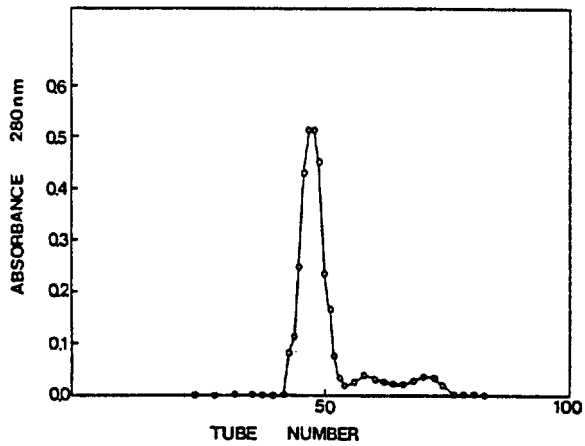

Figure 1. Sephadex G-50 chromatography of PMS-subtilisin. The major peak is PMS-subtilisin.

Measurement of Aggregation. In order to estimate the rapid process of aggregation (or precipitation), turbidities were measured by reading the optical density (O.D.) at 600 nm.

Determination of Ammonia. The time course of evolution of ammonia during thermoinactivation of PMS-subtilisin was determined by incubating samples of the enzyme in sealed ampules at 90 °C for various periods of time. The amount of dissolved ammonia was measured enzymatically with glutamate dehydrogenase.[16]

Results and Discussion

One of the ideal approach to stabilization of an enzyme is to identify the mechanism of its inactivation and then to design the specific stabilizing methods which can prevent the inactivation process from taking place. In order to find the rational methods for thermal stabilization of subtilisin Carlsberg, the irreversible thermoinactivation of the enzyme was carried out at 90 °C.

Preparation of PMS-subtilisin. Since subtilisin has a proteolytic activity that can lead to a irreversible inactivation by autolysis, the enzyme was inactivated by PMSF. The kinetic parameters for the native subtilisin were examined with substrate BAEE at pH 8.5 and 25 °C. The hydrolytic activity for BAEE was 30 ± 0.1 unit. The K_m and the k_{cat} were 7.5 ± 1 mM and 16.5 sec^{-1} respectively. When the enzyme was treated with PMSF, its activity was lost to 1.5×10^{-2} unit equivalent to 0.5% of original activity. The PMSF treated subtilisin (PMS-subtilisin) was further purified by Sephadex G-50 and an elution profile is presented in Figure 1. To assess its purity, the enzyme was subjected to SDS-PAGE. Most of contaminants from commercial enzyme source were removed, but 25 KD, 15.7 KD (15 KD) and

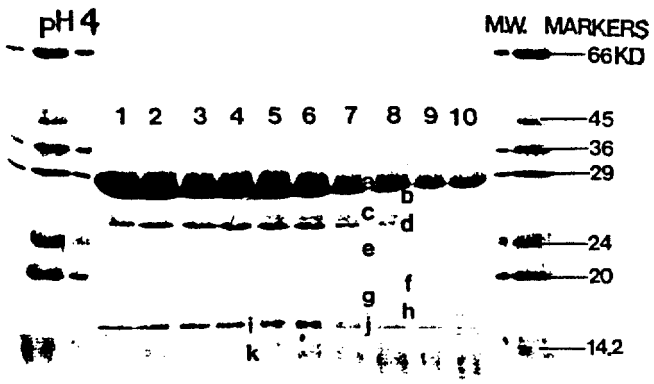

Figure 2. Time dependence of peptide bond cleavage of PMS-subtilisin at pH 4 and 90 °C. Solutions of PMS-subtilisin (0.25 mg/ml were heated for (1) 0 min, (2) 5 min, (3) 10 min, (4) 20 min, (5) 30 min, (6) 1h, (7) 2h, (8) 3h, (9) 4h, and (10) 5h. After TCA treatment, the samples were subjected to SDS-PAGE. New bands are b(27.2 KD), c(25.9 KD), e(22.3 KD), f(19.0 KD), g(17.6 KD), h(16.5 KD), j(15.0 KD), l(12.7 KD). Band d(25 KD), band i(15.7 KD), and band k(13.7 KD) could not be removed from subtilisin in purification step.

13.7 KD (13 KD) could not be removed completely. It seems that these fragments come from the autolysis of subtilisin. A detailed description of the autolysis product has been published elsewhere.[17]

Irreversible Thermoinactivation due to Peptide Bond Cleavage. To elucidate the irreversible mechanisms due to peptide bond cleavage, the protein samples were subjected to SDS-PAGE. The heated samples were centrifuged at 10,000 × g for 5 min and the resulting supernatants were treated with trichloroacetic acid to inhibit the residual proteolytic activity and to concentrate the protein. The gels were stained with Coomassie brilliant blue and analyzed quantitatively by a digital densitometer. The amounts (μg) of proteins in desired bands were compared to calibration curve.

At pH 4, the main band (28.5 KD[18]) disappeared gradually while new distinct bands (M.W. app. 27.2 KD, 25.9 KD, 22.3 KD, 19.0 KD, 17.6 KD, 16.5 KD, 15.7 KD, 15.0 KD, 13.7 KD, 12.7 KD) appeared (Figure 2). According to the Klibanov's result of C-side of Asp cleavage at pH 6-8, we can estimate easily the new bands by the known sequence of subtilisin Carlsberg. Thus, 25.9 KD can result from a cleavage of C-side of Asp-14 and 22.3 KD from a cleavage of Asp-41. A cleavage of C-side of Asp-76 can result in 19.0 KD and that of Asp-172 can give 16.5 KD. The subsequent cleavage of 25.9 KD at Asp-172 can result in 15.0 KD. However 17.6 KD and 15.0 KD which were appeared after 2 hours can not be explained simply by Klibanov's results. Nevertheless it can be assumed that, during the long period of heating, acid hydrolysis could be occurred at free amide group of asparagines and thereby producing new aspartic acids. The subsequent cleavages at the new site could produce 17.6 KD and 15.0 KD. From the densitometer reading of Figure 2, the kinetic constants of irreversible thermoinactivation can be obtained by plotting the amounts of intact subtilisin *vs.* time. The plot presented in Figure 3 shows a straight line with a

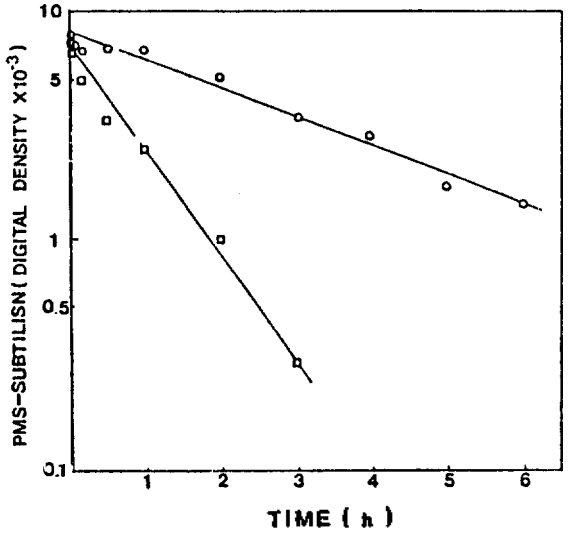

Figure 3. Time course of peptide bond cleavage of PMS-subtilisin at 90 °C. ○, pH 4; □, pH 9.

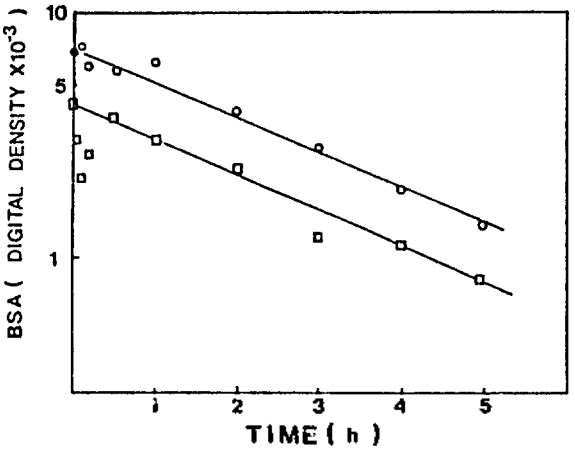

Figure 4. Time course of peptide bond cleavage of BSA in the abssence (○) and presence (□) of subtilisin at pH 4 and 90 °C.

slope of $1.26 \times 10^{-1}h^{-1}$. From the fact that this process followed first-order kinetics, we could postulate that peptide bond cleavage due to autolysis was not included significantly at pH 4. Since autolysis follows second-order kinetics, if it occurs with significant rate. In order to verify this postulation the residual proteolytic activity of subtilisin was examined by heating bovine serum albumine (BSA), in the presence and abscence of subtilisin at 90 °C. The amounts of intact BSA were analyzed by SDS-PAGE (Figure 4). From the scattering points of BSA within 15 min, it can be judged that proteolytic activity of subtilisin was partially present in the initial condition. However the activity seems to diminish fully after 30 minutes. Since the cleavage rate of BSA was same after 30 min whether subtilisin was present or not, yielding a same slope of $1.2 \times 10^{-1}h^{-1}$. This fact designates that after 30 min, peptide-bond cleavage due to autolysis was not involved significantly in the process of irreversible thermoinactivation at pH 4. Therefore it was safely concluded that the new bands stemmed from the acid hydrolysis at the C-side of aspartic acid residues.

At pH 7, any of new distinct bands did not appeared, but

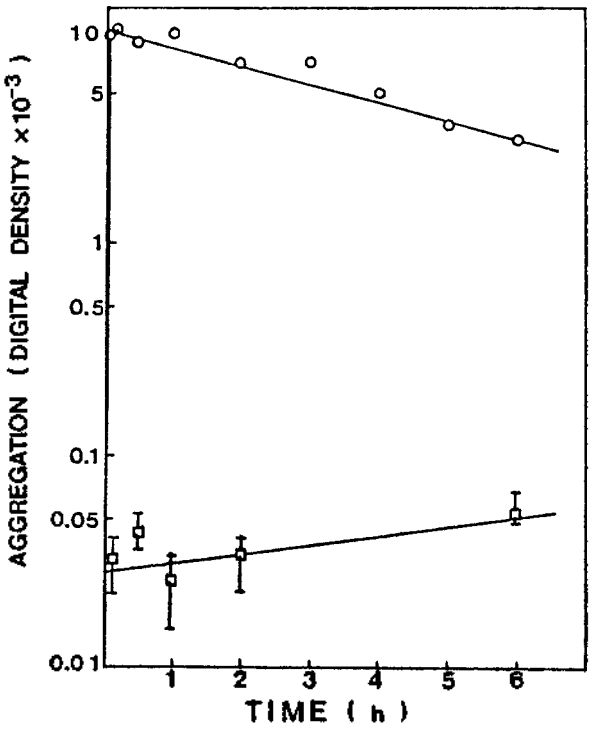

Figure 5. Time course of aggregation of PMS-subtilisin at 90 °C. ○, pH 7; □, pH 4.

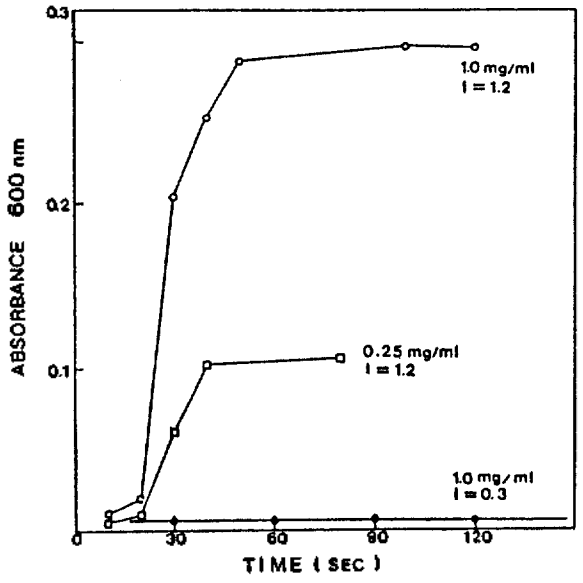

Figure 6. Effect of enzyme concentration and ionic strength on the aggregation of PMS-subtilisin at pH 7

the aggregated enzyme was hydrolyzed slowly and followed first-order kinetics roughly yielding a rate constant of $8.2 \times 10^{-2}h^{-1}$. This value is somewhat higher than that of non-proteolytic enzyme such as α-amylase. Therefore, within the limit of our experiments, it was thought that peptide bond hydrolysis due to autolysis could not be excluded completely in neutral pH region. At pH 9, peptide bond hydrolysis was occurred rapidly (Figure 3). The semilog plotting gave a rate constant of about $4.6 \times 10^{-1}h^{-1}$. The peptide bond hydrolysis under mild alkaline condition might include considerable portion of autolysis. However it could not estimate the relative

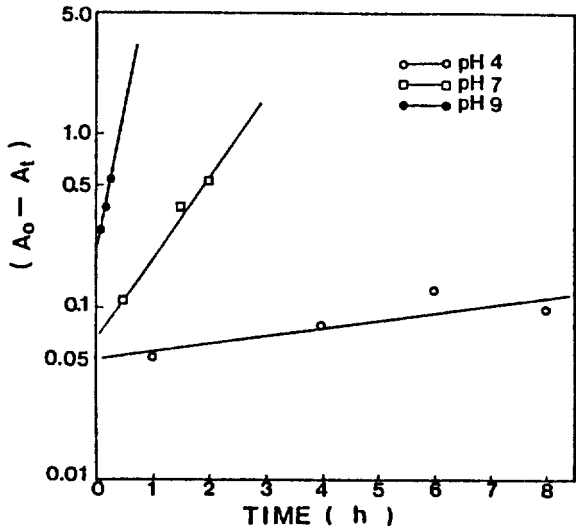

Figure 7. Time course of deamidation of PMS-subtilisin at 90 °C. Absorbance were measured at 334 nm and concentrations of ammonia were determined as described in experimental part.

portion of autolysis to overall hydrolysis because it was hard to obtain the pure PMS-subtilisin used as a control.

Irreversible Thermoinactivation due to Aggregation. In order to elucidate the process of aggregation, the heated samples were centrifuged and precipitates were subjected to SDS-PAGE. In case of fast aggregation, the turbidities of heated samples were directly measured at 600 nm. At pH 4 and 9, the aggregation was very slow (Figure 5), or not detectable within 8 hours. However, at pH 7, this process was relatively fast and completed within 2 min (Figure 5). When the effects of ionic strength and enzyme concentration on the aggregation were examined (Figure 6), it was found that the aggregation process is more sensitive to ionic strength, but little effect of enzyme concentration was observed in the present condition. Therefore to reduce the irreversible thermoinactivation caused by the aggregation at pH 7, it is recommended to use the buffer of low ionic strength.

Irreversible Thermoinactivation due to Deamidation. To determine the kinetic constants of deamidation, released ammonia was analyzed by enzymatic method. At pH 4, the rate of deamidation was considerably slow and the reaction followed first-order kinetics, yielding a rate constant of $4.9 \times 10^{-2}h^{-1}$ (Figure 7). Compared with other processes at pH 4, deamidation is responsible for a relatively small portion of irreversible thermoinactivation of subtilisin. At pH 7 and 9, the rates of deamidation were relatively fast and saturated within 1 or 2 hours (Figure 7). Deamidations at these pHs were also first-order reaction and the rate constants are $4.4 \times 10^{-1}h^{-1}$ and $1.6\ h^{-1}$, respectively. Because of the fast aggregation of subtilisin at pH 7, deamidation at this pH occurred mainly at the aggregated enzyme, therefore deamidation at pH 7 was ruled out from the net direct process of irreversible thermoinactivation. At pH 9, compared with other process, the deamidation seems to be main process for irreversible thermoinactivation. These kinetic data are summarized in Table 1.

Experimental approaches of irreversible thermoinactivation of subtilisin Carlsberg were somewhat different from that of Klibanov's.[6,7] Since subtilisin has a proteolytic activi-

Table 1. Mechanism of Irreversible Thermoinactivation of PMS-subtilisin at 90 °C as a Function of pH

Process	Rate constant (h^{-1})		
	pH 4	pH 7	pH 9
Peptide bond cleavage	1.26×10^{-1}	8.2×10^{-2}	4.6×10^{-1}
Aggregation	4×10^{-2}	16 >	
Deamidation	4.9×10^{-2}	4.4×10^{-1}	1.6

ty that can lead to an irreversible inactivation by autolysis. In Klibanov's procedure, samples of lysozyme (or ribonuclease) that had been heated for various lengths of time at 90 °C were individually reduced,[19,20] desalted, and reoxidized.[20,21] The specific activity of each sample was determined and compared with that of the sample prior to reduction and refolding. Then they assess quantitatively the contribution of processes of which activity can not be recovered by reduction and refolding. However, subtilisin case could not obtain any reproducible data because autolysis of the enzyme occurred excessively at the recovery step. Therefore, subtilisin was modified to block the autolysis by PMSF and thereby the activity of recovered subtilisin could not be measured for quantitative comparison to irreversible inactivation.

The irreversible thermoinactivation of the enzyme at pH 4 was mostly attributed to cleavage of peptide bond at C-side of Asp. However, at pH 7, aggregation which showed sharp dependence on ionic strength was found to be main process. At pH 9, deamidation process was responsible for the most portion of the initial irreversible thermoinactivation.

Acknowledgement. This work was supported by a grant (860614) from the Korea Science and Engineering Foundation.

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