Chem. Pharm. Bull. 25(12)3172—3176(1977)

UDC 577.156.01.02 : 582.282.123.08

Studies on Inactivation of the Semi-alkaline Proteinase from *Aspergillus melleus*[1]

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(Received March 17, 1977)

The thermal inactivation of the semi-alkaline proteinase from *Aspergillus melleus*, proceeded following the first order kinetics. The activation energy for the thermal inactivation was calculated to be 53 kcal per mol. Autolysis takes place directly proportional with the inactivation and the enzyme was degraded into oligopeptide and or amino acid. It was found that calcium ion, ammonium sulfate, glycerol and saccharose have a stabilizing effect on the semi-alkaline proteinase against the thermal inactivation. In the enzyme, 4—6 mol of calcium ion per mol of protein were found to bound, and by the loss of calcium ion, the enzyme became extremely unstable. In the presence of denaturing agents such as urea, guanidine-HCl and alcohols, the inactivation of the enzyme was accelerated and proceeded with first order kinetics. Activation energy for thermal inactivation decreased in the presence of urea and guanidine-HCl, but unchanged in the presence of alcohols.

From these results, it was concluded that thermal inactivation of the semi-alkaline proteinase proceeded in two steps, that is, denaturation with conformational change and followed degradation with the residual proteinase. The rate limiting step was the denaturation.

Keywords——proteinase; *Aspergillus melleus*; inactivation; first order kinetics; autolysis; calcium ion; denaturation; stability

Kinetic studies on the inactivation of enzyme have been published,[3] although most of the previous publications treated the reaction rate in first order kinetics without taking sufficient consideration. For understanding of the mechanism of the inactivation, it is of importance to study factors affecting kinetics of inactivation. Since the condition of denaturation could be defined only during the initial period of the reaction, initial concentration of protein seemed to be important for the understanding the process of denaturation. In the cases of inactivation of ATP-ase[4] and α-amylase,[5] the kinetic order which was calculated from the intial rate at various concentration of the enzyme was differed from that obtained from following the time course of the remaining activity at an enzyme solution. Inactivation of proteinase was considered to be different from that of other enzyme, as autolysis affected the kinetics of inactivation. Actually the inactivation of pepsin[6] was complicated and the kinetic order changed with the enzyme concentration.

In this paper, thermal inactivation of the semi-alkaline proteinase purified from *Aspergillus melleus*[7] was investigated kinetically, and influences of calcium ion and denaturating agents were studied. The autolysis of the enzyme during inactivation was also discussed.

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Experimental

Enzyme——The semi-alkaline proteinase was purified from *Aspergillus melleus* by the method of preceding papers.[7] Before use, the enzyme was rechromatographed on a column of Sephadex G-50 which was previously equilibrated with 10 mM citrate buffer (pH 6.5) containing 1 mM $CaCl_2$, and the eluted active fractions were concentrated by ammonium sulfate precipitation.

Assay of Proteolytic Activity——The proteolytic activity of the semi-alkaline proteinase was assayed according to the method of Nomoto and Narahashi[8] with use of casein as the substrate.

Assay of Protein and Calcium——Protein was assayed by the method of Lowry, *et al.*[9] or by examining the absorbance at 280 nm. The concentration of calcium was measured by an atomic absorption spectrophotometer (Hitachi, model 303) with Ca-Hollow cathod lamp at 422.7 nm.

Gel Filtration on Sephadex G-25——For the analysis of autolysis products, gel filtration chromatography was carried out. The sample (1 ml) was added in 6 M guanidine-HCl (3 ml) and the mixture was applied onto the column (1.6 × 50 cm) of Sephadex G-25 which was previously equilibrated with 8 M urea. Elution was carried out with the same solution at a constant flow rate of 10 ml/hr.

Results

Thermal Inactivation

To make sure the inactivation kinetics of the semi-alkaline proteinase, thermal inactivation was investigated. The enzyme dissolved in McIlvaine buffer (pH 7.0) at a concentration of 1 mg/ml was incubated at various temperatures. The remaining activity was assayed and semi-logarithmic ploting was made. As the result, linear semi-logarithmic ploting for the time elapse was obtained, suggesting that the thermal inactivation of the semi-alkaline proteinase followed first order kinetics with respect to the incubation time. Essentially the same linear semi-logarithmic inactivation was observed between pH 3 and 9, and the enzyme was most stable at pH 6—7. Then the thermal inactivation of the enzyme of different concentration was investigated at 50°. As shown in Fig. 1, first order kinetics of inactivation of the enzyme was observed in every case studied, although the enzyme became unstable with dilution. To find the reason for the decrease of stability with the enzyme concentration, denatured protein was added in the enzyme solution. As the result, the enzyme became stable and it was presumed that some low molecular weight substance containing in the enzyme solution should affect on the stability. The enzyme solution was dialyzed with McIlvaine buffer containing 0.1 M $CaCl_2$, and diluted with the dialyzed buffer solution. The results of thermal inactivation test were shown in Fig. 1. There was no difference in the inactivation rate constant between the various concentrations of enzyme solution, and it became definite that the thermal inactivation also proceeded in the first order kinetics with respect to the concentration.

Autolysis

Kinetic studies were carried out on the role of autolysis in thermal inactivation of the semi-alkaline proteinase. The enzyme dissolved in McIlvaine buffer (pH 7.0) at 2 mg/ml was incubated at 50°. After incubation for various periods, 1 ml of the enzyme solution was poured into 3 ml of 6 M guanidine-HCl solution and mixed well. The mixture was applied onto a column of Sephadex G-25 and eluted with 8 M urea. As shown in Fig. 2, autolysis proceeded with the incubation time and degraded products with medium molecular weight appeared scarcely but most of them degraded to oligopeptides and amino acids. Further, the peak area of the high molecular component was dierctly proportional to the remaining enzymic activity. Consequently, it seemed that the high molecular component arised from the native enzyme.

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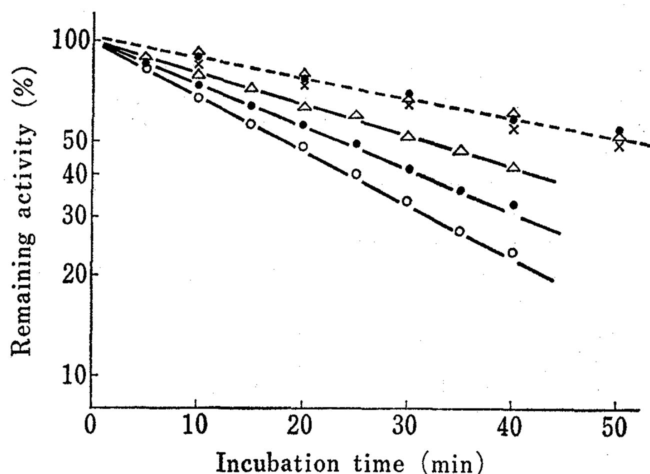

Fig. 1. Thermal Inactivation of the Semi-alkaline Proteinase in Various Solutions at 50°

The enzyme was dissolved in McIlvaine buffer (pH 7.0) at 1 mg/ml (—●—) and 0.1 mg/ml (—○—), and in McIlvaine buffer (pH 7.0) containing 0.9 mg/ml of denatured semi-alkaline proteinase at 0.1 mg/ml (—△—). The semi-alkaline proteinase was dialyzed in McIlvaine buffer (pH 7.0) containing 1 mM $CaCl_2$ and diluted with the buffer at 1 mg/ml (---△---), 0.1 mg/ml (---●---) and 0.01 mg/ml (---×---).

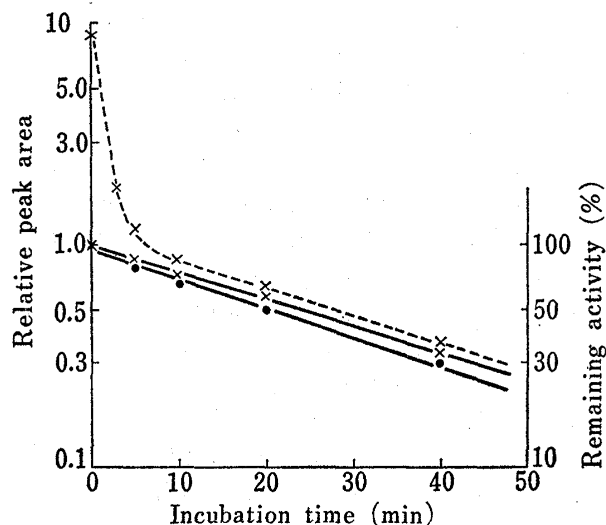

Fig. 3. Degradation of the High Molecular Component during the Thermal Inactivation of the Semi-alkaline Proteinase

The solution of the enzyme was incubated at 50° in the presence (×) and absence (●) of denatured semi-alkaline proteinase by guanidine-HCl. The remaining proteolytic activity (——) and the high molecular component in the chromatogram on Sephadex G-25 (------) were assayed.

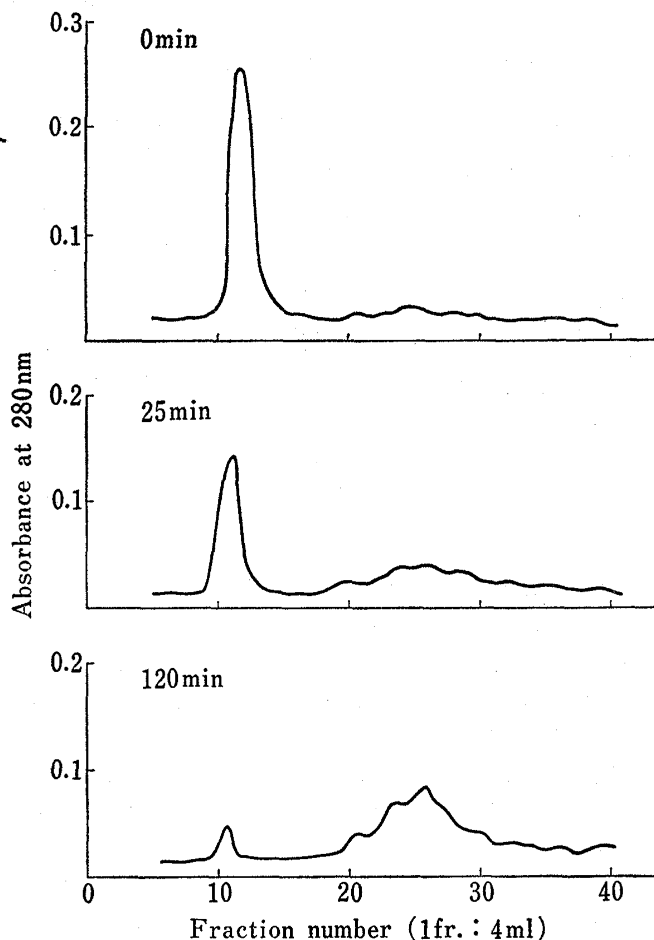

Fig. 2. Column Chromatogram of Thermal Inactivated Semi-alkaline Proteinase on Sephadex G-25

The enzyme was incubated at 50° for various periods of time. After the denaturation by 6 M guanidine-HCl, the protein was chromatographed on a column of Sephadex G-25 which was previously equilibrated with 8 M urea.

The gel filtration chromatograms of the thermal inactivated enzymes in the presence and absence of denatured semi-alkaline proteinase were compared. The denatured semi-alkaline proteinase was prepared as follows; After the treatment with 6 M guanidine-HCl, the solution was chromatographed on Sephadex G-25 and the fraction with high molecular weight was used in the experiment. From the results shown in Fig. 3, it has become apparent that degradation of the denatured protein proceeded very rapidly than that of native protein, and thermal inactivation of the semi-alkaline proteinase was slightly affected by the presence of denatured semi-alkaline proteinase. Also, in this experiment, degradation products with medium molecular weight were not found.

Effect of Calcium Ion

It was found that various compounds such as ammonium sulfate, glycerol, saccharose and $CaCl_2$ had a stabilizing effect of the semi-alkaline proteinase against thermal inactiva-

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Table I. Content of Calcium in the Semi-alkaline Proteinase Dialyzed with Various Solutions and the Rate of Thermal Inactivation

Dialysis solution	Concentration of semi-alkaline proteinase in visking tude (M)	Calcium bound to semi-alkaline proteinase (mol/mol protein)	Rate constant of inactivation (min^{-1})
1 mM EDTA	2.4×10^{-6}	3.5	7.1×10^{-2}
Deionized water	1.0×10^{-5}	4.9	2.4×10^{-2}
0.01 mM $CaCl_2$	1.7×10^{-5}	4.5	1.7×10^{-2}
0.1 mM $CaCl_2$	2.1×10^{-5}	5.8	6.8×10^{-3}

About 10 ml of the semi-alkaline proteinase solution (1 mg/ml) was dialyzed in the visking tube with various solutions (3 l) for 3 days at 4°, and the content of calcium was assayed with an atomic absorption spectrophotometer. The thermal inactivation test was carried out at 50°.

Table II. Rate of Inactivation of the Semi-alkaline Proteinase in the Presence of Various Reagents

Concentration (M)	Rate constant of inactivation (min^{-1})						
	None	1	2	3	4	6	8
Urea	2.7×10^{-3}	—	9.1×10^{-3}	—	2.3×10^{-2}	7.1×10^{-2}	1.3×10^{-1}
Guanidine-HCl	—	1.5×10^{-2}	4.8×10^{-2}	1.1×10^{-1}	1.4×10^{-1}	—	—

Concentration (%)	20	30	40	50	60	70	80
Methanol	—	1.3×10^{-2}	3.4×10^{-2}	8.7×10^{-2}	2.4×10^{-1}	2.1×10^{-1}	1.9×10^{-1}
Ethanol	8.7×10^{-3}	2.5×10^{-2}	7.8×10^{-2}	2.6×10^{-1}	1.8×10^{-1}	1.1×10^{-1}	9.2×10^{-2}
Propanol	3.6×10^{-2}	2.1×10^{-1}	1.8×10^{-1}	1.3×10^{-1}	7.9×10^{-2}	5.5×10^{-2}	3.9×10^{-2}

The enzyme was dissolved in McIlvaine buffer (pH 7.0) at a concentration of 1 mg/ml. The thermal inactivation was carried out at 45°.

tion. Further studies were carried out about the role of calcium. The semi-alkaline proteinase solution was dialyzed with various solutions, and the concentration of calcium ion at the inside and outside solution of visking tube was assayed with an atomic absorption spectrophotometer. The results were summarized in Table I. From the results, it was found that the semi-alkaline proteinase contained 4—6 mol of calcium ion per mol and the loss of calcium ion brought a drastic unstability on the enzyme.

Effect of Various Agents

Kinetic studies on the inactivation by urea, guanidine-HCl and alcohols were carried out. The inactivation of the semi-alkaline proteinase in the presence of the reagent at 45° was proceeded with first order reaction and the results were summarized in Table II. The rate of inactivation by urea and guanidine-HCl increased with the concentration of the agent. On the other hand, the inactivation by alcohols was differed from that of urea and guanidine-HCl. The inactivation rate became maximum at a medium concentration of alcohol, and the concentration became lower with the length of carbon chain of the alcohol. Comparison

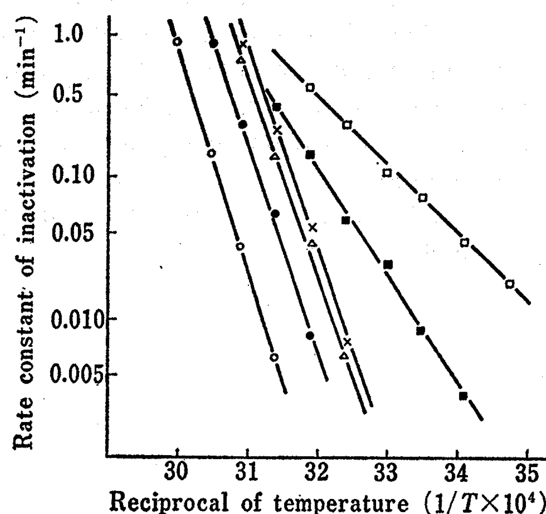

Fig. 4. Arrhenius Plot for the Thermal Inactivation of the Semi-alkaline Proteinase in Various Solutions

○; water, ●; methanol (45%), △; ethanol (45%), ×; propanol (45%), ■; urea (8 M), □; guanidine-HCl (5 M).

of the activation energy for the inactivation of the enzyme in various conditions was carried out with use of the Arrhenius plot. From the results shown in Fig. 4, it was found that the activation energy in buffer solution at pH 7.0 was 53 kcal, and urea and guanidine-HCl decreased the activation energy, but alcohols did not change.

Discussion

The semi-alkaline proteinase[7] which was produced by *Aspergillus melleus* was characterized as the enzyme having a optimum pH of enzymic action in semi-alkaline region and distinguished from neutral and alkaline proteinases. The enzyme is not a metal enzyme but a serine proteinase, and it lacks disulfide bond like thermolysin[10] and neutral protease of *Bacillus subtillis*.[11]

The thermal inactivation of the semi-alkaline proteinase dissolved in water proceeded in first order rate kinetics with respect to the incubation time, but rate constants of various concentrations were not same. On the other hand, the enzyme solution which was dialyzed with a buffer solution and diluted with the same solution was inactivated according to the first order equation. Therefore it was concluded that the thermal inactivation of the enzyme followed first order kinetics essentially and certain substances of low molecular weight contained in the enzyme affected on the rate of thermal inactivation. By the addition of calcium ion in the buffer solution, the enzyme became stable, and in this condition, the enzyme contained 4—6 mol of calcium per mol. On the other hand, the calcium content became below 4 mol by dialysis with water or by addition of EDTA the enzyme became very unstable. These properties were similar to those of thermolysin and neutral protease from *Bacillus subtilis*.[12] The autolysis of the semi-alkaline proteinase was investigated, and it was found that autolysis was proceeded directly proportional to the inactivation and the enzyme was degraded into oligopeptides and or amino acids. These results were agreeable to the fact that the degradation of the denatured enzyme by the semi-alkaline proteinase was extremely faster than that of native one. The mechanism of thermal inactivation of the semi-alkaline proteinase can be summarized as follows; The first step was the denaturation or modification with conformation change, then followed by proteolysis that was catalyzed by the remaining enzyme. The former step was the rate limiting, and the rate may be accelerated by heat, H^+, OH^- and various reagents such as urea, guanidine-HCl and alcohols, on the other hand, the rate should be depressed by agents such as glycerol, saccharose, ammonium sulfate and Ca^{2+}. If the autolysis determined the inactivation rate, the equation must differ from the first order kinetics because the autolysis is intermolecular reaction. Further, the activation energy for the inactivation was estimated to be 53 kcal/mol and the value differed significantly from that of proteolysis (6.1 kcal/mol). The proposed mechanism is also well agreeable to the fact many proteins[13] denatured in the first step and followed by proteolysis bustly.

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