Inactivation Kinetics of Enzyme Pharmaceuticals in Aqueous Solution

Sumie Yoshioka,[1,2] Ken-ichi Izutsu,[1] Yukio Aso,[1] and Yasushi Takeda[1]

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The kinetics of enzyme inactivation in aqueous solution of neutral pH were studied for α-chymotrypsin, bromelain, and kallikrein. Inactivation of α-chymotrypsin and bromelain followed simple first-order kinetics, and the rate constant obtained conformed to the Arrhenius relationship. Kallikrein, however, presented more complicated kinetics of inactivation, which could be described by a kinetic expression combining a reversible and an irreversible pathway. Nonlinear regression analysis suggested that the rate constants conform reasonably well to the Arrhenius relationship. The results suggest that inactivation of enzymes in aqueous solution can be modeled even if the profile is complicated and that the inactivation rates can be predicted based on the relationship between the parameter estimates and temperature.

KEY WORDS: enzyme; inactivation; degradation; kinetics; α-chymotrypsin; bromelain; kallikrein.

INTRODUCTION

The number of preparations of proteins as medicinal agents on the market has been increasing. These include parenteral preparations of proteins produced by advanced protein technology (1) as well as classical preparations such as solid dosage forms of digestive enzymes. In addition, various sophisticated dosage forms of proteins are being developed (2–4).

The stability evaluation of these drug products is a challenge because proteins present more complicated degradation mechanisms than small organic pharmaceuticals. Although proteins are known to degrade via various chemical and physical pathways (5), the degradation kinetics of protein preparations from the viewpoint of stability prediction or evaluation have been reported for only a few cases (6–11). The available kinetic data are inadequate to determine the dependence of protein degradation rates on temperature and the utility of accelerated stability testing for stability prediction.

For stability prediction of protein preparations, it is necessary to clarify the kinetics of protein degradation. Since enzymes have been found to be thermostable in the dry state (12,13), and it has been suggested that water directly or indirectly participates in enzyme thermoinactivation processes (13), kinetic data of protein degradation in aqueous solution should provide the basic information necessary to understand degradation in preparations of interest. Very little work, however, has been done on the kinetics of protein degradation in aqueous solution (13,14). We therefore studied degradation kinetics of three peptidases (α-chymotripsin, bromelain, and kallikrein) in aqueous solution at neutral pH.

EXPERIMENTAL

Materials

Bovine pancreas α-chymotrypsin (chymotrypsin A4) was purchased from Boehringer Mannheim Yamanouchi Co. (Tokyo), and bromelain from Mochida Pharmaceutical Co. (Tokyo). Kallikrein (Circuletin, lyophilized with lactose and sodium chloride) was purchased from Teikoku Zoki Pharmaceutical Co. (Tokyo). Enzymes were used without further purification. *N*-Acetyl-L-tyrosine ethyl ester (ATEE), benzoyl-L-arginine ethyl ester hydrochloride (BAEE HCl), and benzoyl-L-arginine (BA) were purchased from Aldrich (Milwaukee), Sigma (St. Louis), and Tokyo Kasei Co. (Tokyo), respectively. Casein (nach HAMMARSTEN) was purchased from Merck, and tyrosine was JP XI reference standard.

Kinetic Studies

α-Chymotrypsin was dissolved in 0.001 *N* hydrochloric acid to make a 0.4-mg/ml solution. An appropriate aliquot of the solution was diluted with 50 m*M* phosphate buffer to make a 10-μg/ml solution. The enzyme concentration corresponded to 1.5 μmol ATEE/min/ml. The phosphate buffer used was pH 7.4 and ionic strength 0.15 (adjusted with NaCl), and the buffer was prewarmed at 30, 35, 40, or 45°C prior to addition of the α-chymotrypsin. The sample solutions were then stored at 30, 35, 40, or 45°C in a thermostatic water bath, and a 0.1-ml aliquot of the solution was taken at appropriate intervals for the activity assay.

Similar methods were used to study bromelain inactivation. Bromelain was dissolved in phosphate buffer, pH 7.4, to make a 1.25-mg/ml solution. After filtering through a 0.45-μm membrane to remove a small amount of insoluble material, an appropriate aliquot of the solution was added to a buffer solution preequilibrated at 45, 50, 55, or 60°C to give a 0.125-mg/ml solution (the enzyme concentration corresponded to 50 μg tyrosine/min/ml). Solutions were stored at each temperature, and a 1-ml aliquot of each solution was taken at appropriate intervals for the activity assay.

Kallikrein was dissolved in phosphate buffer, pH 7.4, to make a 5-μmol BA/min/ml solution. The buffer was prefiltered through a 0.45-μm membrane to avoid microbial contamination since the experimental duration was relatively long for kallikrein. The sample solutions were stored at 45, 50, 55, or 60°C, with 0.5-ml aliquots being removed for the activity assay at appropriate intervals.

Activity Measurement

The activity of α-chymotrypsin was determined by the USP method (15), using ATEE as a substrate.

The activity of bromelain was determined by measuring the formation rate of the decomposition product of casein (16). The sample solution was incubated with 5 ml of a 6-mg/ml casein solution (in 50 m*M* phosphate buffer, pH 7.0)

[1] National Institute of Hygienic Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158, Japan.

[2] To whom correspondence should be addressed.

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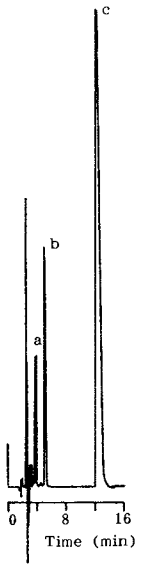


Fig. 1. Typical chromatographic pattern for activity determination of kallikrein. Benzoyl-L-arginine (a), *p*-aminobenzoic acid (internal standard) (b), and benzoyl-L-arginine ethyl ester (c).

at 37°C for 10 min. A 5-ml aliquot of 0.11 *M* trichloroacetic acid solution (in 0.22 *M* acetate buffer) was then added to the sample solution, and the mixture was stored at 37°C for 40 min. After filtration through a 0.45-μm membrane, the absorbance of the filtrate was measured at 275 nm using tyrosine as a standard.

A high-performance liquid chromatographic (HPLC) method was employed to determine the activity of kallikrein. The procedure recently described by Pietta *et al.* (17) was modified as follows. The sample solution (0.5 ml) was added to 2.5 ml of the substrate solution (0.2 *M* BAEE HCl in 0.1 *M* Tris–HCl buffer, pH 8), preequilibrated at 30°C, and stored at 30°C. Aliquots (200 μl) of the solution were taken at 30-sec intervals (five points) and added to 2 ml of 0.05 *N* HCl containing 0.3 mg/ml *p*-aminobenzoic acid (internal standard) to stop the reaction. The decomposition product (BA) of the substrate in the reaction solution was determined by HPLC. The method employed a Toso TSK-ODS80TM

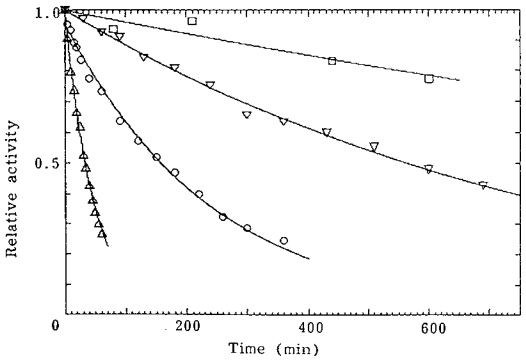


Fig. 2. Profiles of α-chymotrypsin inactivation in pH 7.4 aqueous solution. The initial enzyme activity was 1.5 μmol ATEE/min/ml. The solid lines were calculated from the parameters obtained by nonlinear regression analysis according to a first-order kinetic expression. □, 30°C; ▽, 35°C; ○, 40°C; △, 45°C.

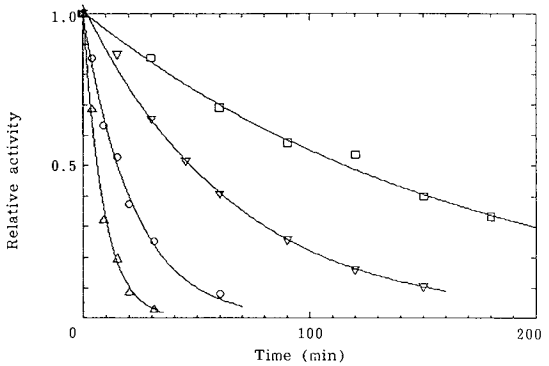


Fig. 3. Profiles of bromelain inactivation in pH 7.4 aqueous solution. The initial enzyme activity was 50 μg tyrosine/min/ml. The solid lines were calculated from the parameters obtained by nonlinear regression analysis according to a first-order kinetic expression. □, 45°C; ▽, 50°C; ○, 55°C; △, 60°C.

packed column (4.6 mm × 15 cm) (Tokyo) and a mobile phase of 10 m*M* phosphate buffer (pH 2.8)/acetonitrile (85/15) with a flow rate of 1.0 ml/min. The eluate was monitored at 254 nm. The HPLC equipment consisted of a Hitachi Model 655 system (Tokyo), a Toso Model AS-8000 autosampler (Tokyo), and a Shimadzu Model C-R3A computing integrator (Kyoto). The amount of BA formed per second was calculated from the peak height (Fig. 1).

RESULTS AND DISCUSSION

Figures 2 and 3 show the profiles of α-chymotrypsin and bromelain inactivation, respectively, in aqueous solution at various temperatures. The activity ratio to the initial value is plotted against time. Inactivation appeared to follow first-order kinetics for both enzymes. The solid lines shown in the figures were calculated from the parameters obtained by nonlinear regression analysis according to a first-order kinetic expression. Figures 4 and 5 show the Arrhenius plots of the apparent first-order rate constants obtained for α-chymotrypsin and bromelain, respectively. The activation energies calculated by assuming linearity of the plots were 51.4 kcal/mol (α-chymotrypsin) and 41.7 kcal/mol (bromelain).

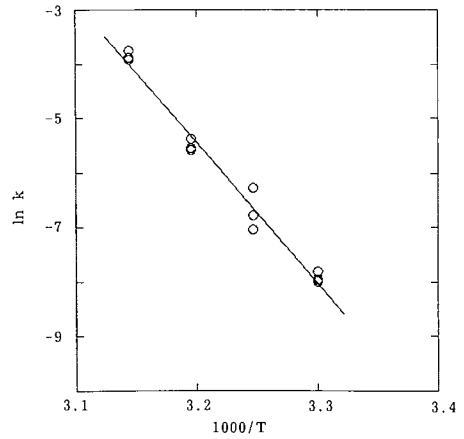


Fig. 4. Arrhenius plots of apparent first-order rate constants obtained for α-chymotrypsin inactivation (pH 7.4).

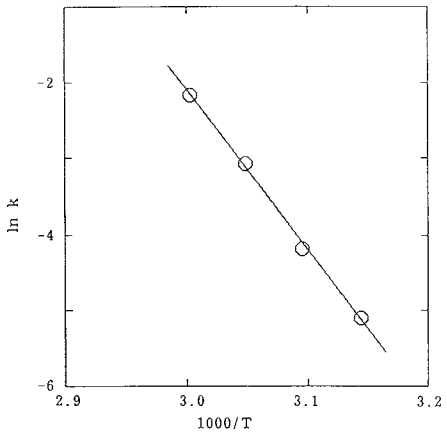


Fig. 5. Arrhenius plots of apparent first-order rate constants obtained for bromelain inactivation (pH 7.4).

Protein degradation in aqueous solution has been described by a first-order expression in most of the studies published to date (13,14). Even the solid-state degradation of proteins has been reported to follow first-order kinetics (9–11). In this study, α-chymotrypsin and bromelain were found to degrade in a similar pattern. Information on the activation energy of protein degradation is very limited, though some is available for solid-state degradation (7–9,11). The apparent activation energies calculated for α-chymotrypsin and bromelain inactivation in the present study were much larger than those reported for human interferon (ca. 20 kcal/mol) (9) and horse serum cholinesterase (ca. 25 kcal/mol) (11) degradation in the solid state.

In contrast to the simple first-order kinetics observed for α-chymotrypsin and bromelain inactivation, kallikrein degradation followed more complicated kinetics. Figure 6 shows profiles of kallikrein inactivation at 50°C, which was analyzed by the four kinetic models shown in Scheme I. Models 1 and 2 represent an irreversible first-order inactivation, and a reversible first-order inactivation, respectively. In Model 3, kallikrein is assumed to have an irreversible degradation pathway as well as a reversible pathway. Model 4 assumes that the kallikrein sample used includes two different active enzymes which degrade independently according to first-order kinetics. The solid lines in Fig. 6 were calculated from the parameters obtained by nonlinear regression analysis according to each kinetic expression. The sums of squared deviations, when the data were fitted to Models 1, 2, 3, and 4, were calculated to be 0.0561, 0.0235, 0.0054, and 0.0055, respectively. Inactivation of kallikrein can be described by Model 3 or 4 with a significantly smaller sum of squared deviation than Model 1 or 2. No significant difference in the sum of squared deviation was observed between Model 3 and Model 4, but Model 4 provided unreasonable parameter estimates. The percentage of the enzyme undergoing the faster inactivation process varied with temperature. This is unreasonable and excludes Model 4 from being a reasonable description of the inactivation data of kallikrein. Figure 7 shows profiles of kallikrein inactivation at various temperatures, analyzed by the Model 3 kinetic expression. The solid lines represent the calculated amounts of native enzyme and inactive enzyme, D. Model 3 provides an

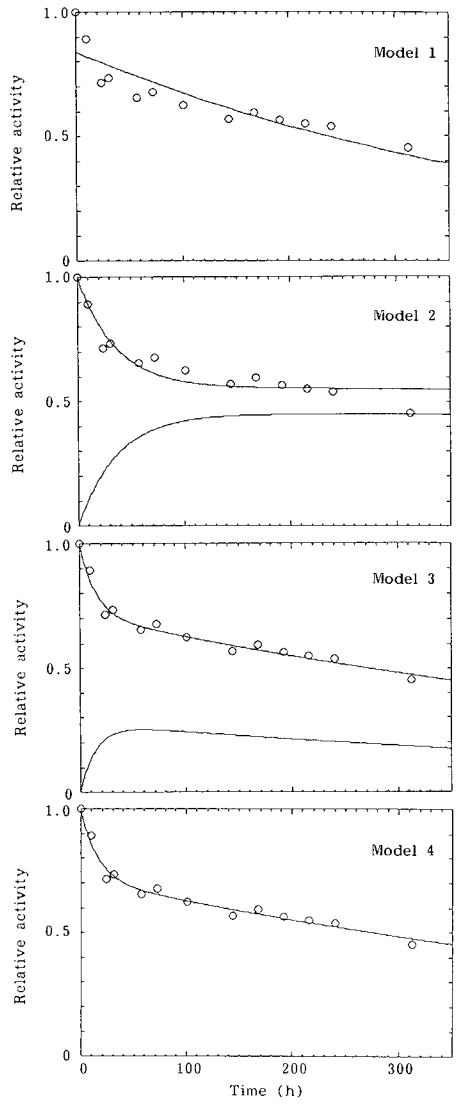
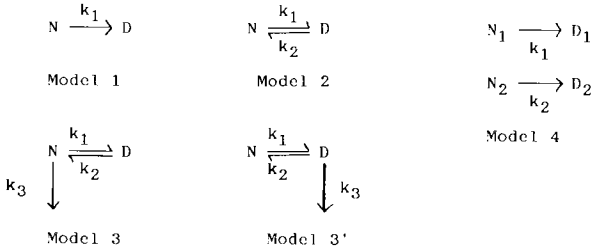


Fig. 6. Profiles of kallikrein inactivation at 50°C in pH 7.4 aqueous solution. The solid lines were calculated from the parameters obtained by nonlinear regression analysis according to Models 1, 2, 3, and 4 (see text).

excellent fit of the data. Figure 8 shows the Arrhenius plots of the estimated first-order rate constants. The activation energies calculated for k_1, k_2, and k_3 by assuming the linearity of the plots were 71.4, 57.0, and 114.6 kcal/mol, respectively. Since k_3 is smaller than k_2 at lower temperature and the activation energy of k_3 is larger than that of k_2, the Ar-



Scheme I. Kinetic models for kallikrein inactivation N and D represent intact enzyme and inactive enzyme, respectively.

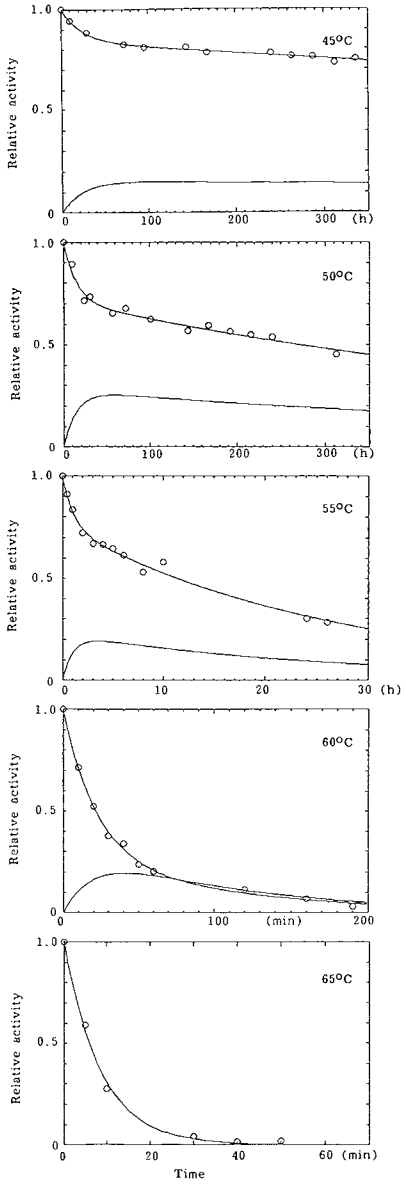


Fig. 7. Profiles of kallikrein inactivation in pH 7.4 aqueous solution at various temperatures. The solid lines were calculated by nonlinear regression analysis according to Model 3.

rhenius plots obtained can explain why the activity appears to approach equilibrium at lower temperature and why the inactivation time profile approaches an apparent first-order pattern as temperature increases (Fig. 7).

Model 3 seems to be the best of the considered models to describe the inactivation profile of kallikrein, because it gives reasonable parameter values and a small sum of squared deviations. Though Model 3 assumes that kallikrein degrades via a reversible pathway as well as an irreversible pathway, it is also possible to assume that kallikrein degrades reversibly to an inactive form, D, which is susceptible to further degradation (Model 3′ in Scheme I). Models 3 and 3′ are indistinguishable based solely on the activity versus time data obtained in the present study. Determination of the inactive form, D, of the enzyme would be required to distinguish between these two models.

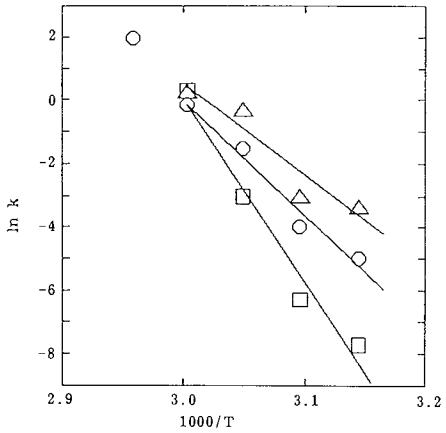


Fig. 8. Arrhenius plots of apparent first-order rate constants obtained for kallikrein inactivation (pH 7.4). ○, k_1; △, k_2; □, k_3.

In conclusion, the kinetics of enzyme inactivation in aqueous solution were studied for α-chymotrypsin, bromelain, and kallikrein. Inactivation of α-chymotrypsin and bromelain was found to follow simple first-order kinetics, and the rate constant obtained appeared to conform to the Arrhenius relationship, suggesting that the inactivation rate can be predicted by interpolating the relationship. Kallikrein, on the other hand, presented more complicated inactivation kinetics, which could be described by a kinetic expression combining a reversible and an irreversible pathway. The rate constants obtained by nonlinear regression analysis provided a reasonable Arrhenius relationship. The results obtained suggest that inactivation of enzymes in aqueous solution can be modeled even if the profile is complicated and that the dependence of kinetic parameters on temperature can be determined. The enzyme inactivation considered here revealed very large values of activation energy. Though the wide range of activation energies observed for protein degradation cannot be explained unless the degradation mechanisms are known, it may be possible to predict enzyme inactivation rates based on the relationship between those parameters and temperature.

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