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ARTICLE

A comparative study of the conformational stabilities of trypsin and α-chymotrypsin

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ABSTRACT A comparative study was performed on the conformational stabilities of trypsin and -chymotrypsin. At 45°C, trypsin was most stable at pH 3, while the highest stability of -chymotrypsin was observed at pH 5. With both ester and amide substrates, trypsin displayed activation at pH 3. In the case of -chymotrypsin, activation was detected at pH 5 only with the amide substrate. The time curves of heat inactivation were complex. For both enzymes, autolysis proceeded with the highest velocity at pH 8. The results obtained on -chymotrypsin suggested consecutive reactions: the first step, heat denaturation of the protein, is followed by digestion of the damaged molecules. **Acta Biol Szeged 45(1-4):43-49 (2001)**

KEY WORDS

trypsin
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conformational stability
autolysis
pH effect

Trypsin and -chymotrypsin are well-known serine proteases (Desnuelle 1971; Keil 1971; Cohen et al. 1981; Journak and McPherson 1987). The serine proteases exhibit structural and chemical similarities, but their specificities are different (Polg r 1989).

According to early observations, trypsin is stable at pH 3 at low temperatures for weeks. It can be reversibly heat denaturated (Lazdunski and Delaage 1965). Lazdunski and Delaage (1967) investigated the effect of pH on the temperature-induced reversible denaturation of bovine trypsin. D'Albis (1970) conducted a thermodynamic study on the reversible thermodenaturation of trypsin in the pH range 1.0-3.4. The conformation of trypsin is well ordered between pH 7 and 8, but is considerably less ordered at more acidic or alkaline pH values. Both enzymes are susceptible to autolysis. Chymotrypsin A is most stable at pH 3, but even at this pH autolysis proceeds, although very slowly. At pHs lower than 3 or higher than 10, the enzyme undergoes conformational changes (Walsh and Wilcox 1970).

The conversion of trypsin to chymotrypsin and vice versa by site-directed mutagenesis is a model for protein engineers (Gr f et al. 1987; Heldstrom et al. 1992). Site-directed mutagenesis could modify the conformational stabilities of the enzyme derivatives. For an appraisal of the stability changes induced, a kinetic re-evaluation of the conformational stabilities of the parent enzymes appeared reasonable.

Heat denaturation experiments comprise a simple and inexpensive method of investigation of the conformational stabilities of proteins, and are useful for comparative studies, too. The present paper reports results on the heat inactivation of trypsin and -chymotrypsin.

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Materials and Methods

Materials

Bovine pancreas trypsin (EC 3.4.21.4), -chymotrypsin (EC 3.4.21.1), N-benzoyl-L-arginine ethyl ester (BAEE), N-acetyl-L-tyrosine ethyl ester (ATEE), N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) and N-carbobenzoxy-L-phenylalanine-p-nitroanilide (CPPNA) were purchased from Sigma-Aldrich Company (Budapest, Hungary). The specific activities were 40-60 units/mg for -chymotrypsin and 10,000 units/mg for trypsin. All other chemicals were reagent grade products (Reanal, Budapest, Hungary).

Assays of enzyme activities

The activity of trypsin was measured by following the increase in absorbance at 253 nm (Geiger and Fritz 1984) in a reaction mixture (3 ml) containing 46.7 mM Tris/HCl buffer (pH 8.0), 19 mM $CaCl_2$ and 0.9 mM BAEE, the reaction being initiated by the addition of 5 units of enzyme. One unit of enzyme activity was defined as the amount of enzyme that hydrolyses 1 μM of BAEE per min at pH 8.0 and at 25°C. The activity measurements with BAPNA were carried out as follows: the reaction mixture contained 150 mM triethanolamine/HCl (pH 8.0), 15 mM $CaCl_2$, 0.8 mM BAPNA and 5-10 units of trypsin (Erlanger et al. 1961). The amount of p-nitroanilide released was monitored via the increase in absorbance at 410 nm. For the measurement of -chymotrypsin activity, ATEE was used and the changes in absorbance at 237 nm were followed in a reaction mixture (3 ml) containing 40 mM Tris/HCl (pH 8.0), 50 mM $CaCl_2$

and 0.5 mM ATEE (Schwert and Takenaka 1955). The reaction was initiated by the addition of 4.5 units of enzyme. One unit of enzyme activity was defined as the amount of enzyme that catalyses the hydrolysis of 1 µM of ATEE per min at pH 8.0 and at 25ºC. For the activity determination with CPPNA as substrate, the reaction mixture (3.0 ml) contained 50 mM Tris/HCl (pH 8.0), 0.1 mM CPPNA in DMF and 5 units of -chymotrypsin (Delmar et al. 1979). The amount of p-nitroanilide released was monitored via the increase in absorbance at 410 nm.

Stability tests

The pH dependencies of the conformational stabilities of trypsin and -chymotrypsin were studied in the pH range 3-7 by using 0.1 M glycine/HCl buffer (pH 3), 0.1 M acetic acid/NaOH buffer (pH 4-5), 0.1 M citric acid/NaOH buffer (pH 6) and 0.1 M triethanolamine/HCl buffer (pH 7), respectively. Enzyme solutions of 0.1 and 1.0 mg/ml were prepared with the different buffers and incubated for 5 h at various temperatures. Aliquots of 100-200 µl were withdrawn and the residual activities were determined.

Measurements of ninhydrin-positive species

The appearance of ninhidrin-positive substances during heat treatment was followed quantitatively according to the procedure of Moore and Stein (1948).

Results

Effects of pH on stabilities of trypsin and a-chymotrypsin

The pH dependences of the stabilities of trypsin and -chymotrypsin were studied at 45ºC, both ester and amide substrates being used for the determination of residual activities. The protein concentration of the enzyme solution was 1 mg/ml.

For trypsin, similar results were obtained with either BAEE or BAPNA as substrate (Fig. 1), but the loss in amidase activity was somewhat faster, especially in the acidic media. At pH 3, activation was observed with both substrates (18-20% and 16-17%, respectively). Trypsin exhibited the highest stability at this pH. The inactivation was faster in the solutions with pH > 6 than that in the media with lower pHs.

The results obtained with -chymotrypsin are depicted in Fig. 2. Significant differences were found in the stabilities of esterase (ATEE substrate) and amidase (CPPNA substrate) activities, especially at pH < 6. The highest stability of -chymotrypsin was observed at pH 5. At this pH, the esterase activity was preserved for at least 4 h, while with the amide substrate activation of at most 24% was measured. Above pH 6 the inactivation was more rapid than that in the media with lower pHs. At pH 9, the enzyme was practically inactivated during the first 20 min of incubation.

Effects of temperature on stabilities of trypsin and -chymotrypsin

The temperature dependence of the stability of -chymotrypsin was studied at pH 4 in citrate buffer and at pH 7 in phosphate buffer, with ATEE as substrate. The protein concentration was 1 mg/ml. The results are presented in Fig. 3. At pH 4, the enzyme retained about 20% of its starting activity after incubation for 5 h at 50ºC, while at pH 7 the enzyme practically lost all of its activity. At 55ºC, the inactivation was complete during the first 20 min of incubation. In a 1 mg/ml solution, in phosphate buffer (pH 8) at 55ºC, trypsin lost more than 90% of its initial esterase and amidase activities during a 5-min incubation.

Effects of protein concentration on stabilities of trypsin and -chymotrypsin

The effects of 0.1 and 1 mg/ml protein concentrations on the stability of trypsin were studied in Tris/HCl buffer (pH 8) at 55ºC, with both ester (BAEE) and amide (BAPNA) as substrates. After incubation for 2.5 min in the 0.1 mg/ml solution, trypsin had lost 55.6% of its original esterase activity, while in the 1 mg/ml solution only 11.5% of the starting activity was preserved. As regards the amidase activity, after incubation for 5 min in 0.1 mg/ml solution the activity loss was 61.6%, while in 1 mg/ml solution it was 93.2%. In the case of -chymotrypsin, the effects of the protein concentration on the stability were studied at 50ºC at pH 4 (sodium citrate) and pH 7 (potassium phosphate) in 0.1 and 1 mg/ml solutions, with ester (ATEE) and amide (CPPNA) as substrates. The inactivation in the 0.1 mg/ml solution was faster for both types of substrates (Fig. 4).

Autolysis of trypsin and -chymotrypsin

Samples from the heat inactivation experiments were submitted to the ninhydrin test. The time curves of liberation of ninhydrin-positive species from trypsin are shown in Fig. 5. In 1 mg/ml solutions at pH 3 and 4, ninhydrin-positive substances could not be demonstrated, but at pH > 5 the autolysis proceeded rapidly. The highest rate was experienced at pH 8 in Tris/HCl buffer. The process involved at least two phases, a fast and a slower one. At that pH in the 0.1 mg/ml solution, the liberation of ninhydrin-positive substances was not detected at 45ºC and 55ºC.

In 0.1 mg/ml -chymotrypsin solutions, heat-treated at 45ºC and 50ºC and at pH 4 and 7, respectively, ninhydrin-positive species were not liberated. In 1 mg/ml solutions at 45ºC and in the pH range 3-4.5, ninhydrin-positive substances could likewise not be detected. At pH 6, a lag period was followed by the accelerated formation of autolysis products. At pH > 7, the time curves did not exhibit any lag period and the process proceeded rapidly. The maximum velocity was measured at pH 8 in potassium phosphate or Tris/HCl buffer

(Fig. 6). The autolysis involved at least two phases, similarly as for trypsin. At higher temperature (50ºC), the lag period was observed only at pH 4 in the first 25 min of incubation (Fig. 7).

Discussion

Trypsin and -chymotrypsin display close structural similarities. The backbone structure of the two proteases are highly homologous and the homology also extends to the catalytic triad and substrate-binding pocket regions (Steitz et al. 1969; Birktoft and Blow 1972; Polg r 1989). In spite of the structural similarities, however, there are significant differences in their conformational stabilities. In earlier work (Simon et al. 1998), we established that, in miscible polar solvents such as acetonitrile, ethanol and 1,4-dioxane, -chymotrypsin has quite different behaviour from that of trypsin. The differences in the conformational stability are confirmed by the heat treatment experiments. At 45ºC, trypsin is most stable at pH 3, while the highest stability of -chymotrypsin was observed at pH 5. With both ester and amide substrates, trypsin shows activation at pH 3. In the case of -chymotrypsin, activation was detected at pH 5 only with the amide substrate. The time curves of heat inactivation are complex for both enzymes, in consequence of the existence of different

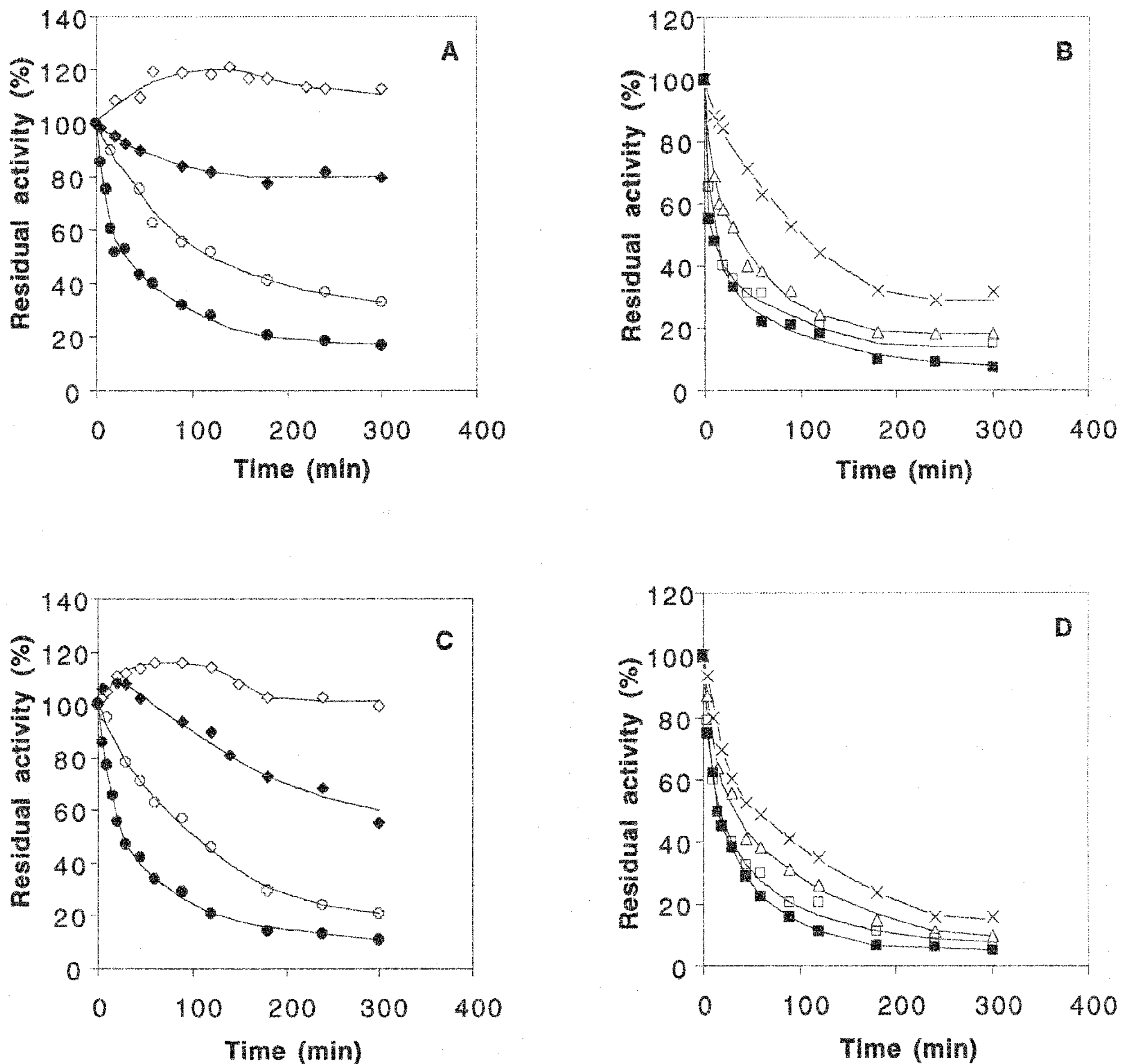

Figure 1. Effects of pH on inactivation of trypsin at 45°C. Protein concentration: 1 mg/ml. Substrates: BAEE (A, B) and BAPNA (C, D). Buffers (0.1 M): citrate ( ) pH 3, ( ) pH 4, ( ) pH 5, (●) pH 6, phosphate (x) pH 6, ( ) pH 7, (□) pH 8; borate (■) pH 9. For details, see text.

molecular forms. The stabilities of the different molecular forms of trypsin are temperature- and pH-dependent (Lazdunski and Delaage 1967). At 20ºC, the acidification of trypsin from pH 8 to pH 0.5 results in the appearance of 3 reversible equilibria. The most important structural change in the alkaline range involves the unmasking of the abnormal tyrosines. This process is reversible, but is followed by an irreversible denaturation. -chymotrypsin can exist in two major conformational states, only one of which is active. Stoesz and Lumry (1978) examined the pH and ionic strength dependence of the transition between the active and inactive forms. At low pH (pH 2.0-6.0), the equilibrium is very dependent on the salt concentration; high salt concentrations effectively stabilize the active conformation. This apparent stabilization is an artifact due to the dimerization of the active form of -chymotrypsin. At pH 6.0-8.0, the dimerization does not occur. At pH > 6, the pH dependence can be described by a two-ionization mechanism at all ionic strengths. The self-association of -chymotrypsin was studied by Pandit and Rao (1974). We suspect that the transient activa-

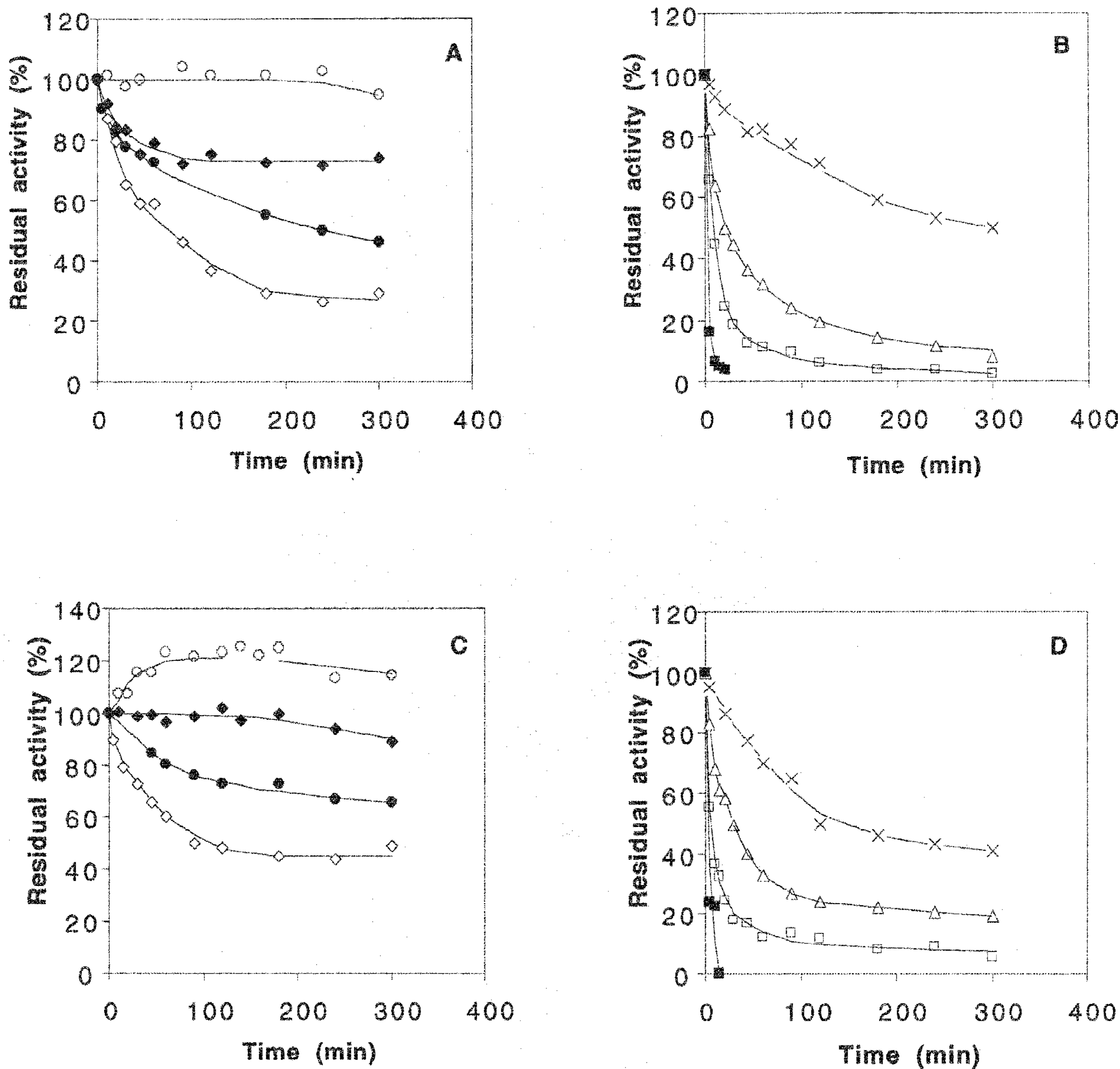

Figure 2. Effects of pH on inactivation of -chymotrypsin at 45°C. Protein concentration: 1 mg/ml. Substrates: ATEE (A,B) and CPPNA (C, D). Buffers (0.1 M): citrate ( ) pH 3, ( ) pH 4, ( ) pH 5, (●) pH 6, phosphate (x) pH 6, ( ) pH 7, (□) pH 8; borate (■) pH 9. For details, see text.

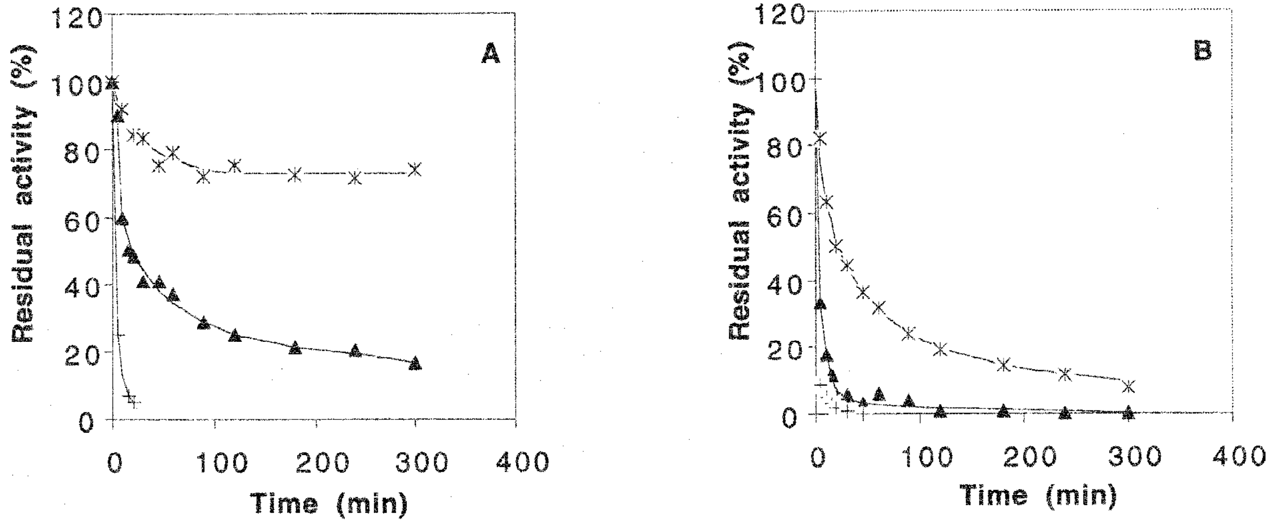

Figure 3. Effects of temperature on inactivation of -chymotrypsin at pH 4 in citrate buffer (A) and at pH 7 in phosphate buffer (B) with ATEE as substrate. Protein concentration: 1 mg/ml. Temperatures: (*) 45°C, (▲) 50°C, (+) 55°C. For details, see text.

tions during the heat treatment stem from the rise of a molecular subform with a higher catalytic activity, but a lower stability.

The autolysis proceeds with the highest velocity at pH 8 for both enzymes. At pH 3 and 4, the liberation of the ninhydrin-positive substances from -chymotrypsin molecules cannot be detected. A similar phenomenon was observed in 0.1 mg/ml solutions (in spite of the fast heat denaturation) at pH 4 and 7 at 45°C and 50°C for -chymotrypsin and at pH 8 at 45ºC and 50ºC for trypsin. The detailed investigation by Kumar and Hein (1970) suggested that the mechanism of autolysis of -chymotrypsin can be explained by an apparent second-order inactivation process. Autodigestion is chemically distinguishable from the process of denaturation. Our experimental results support these findings.

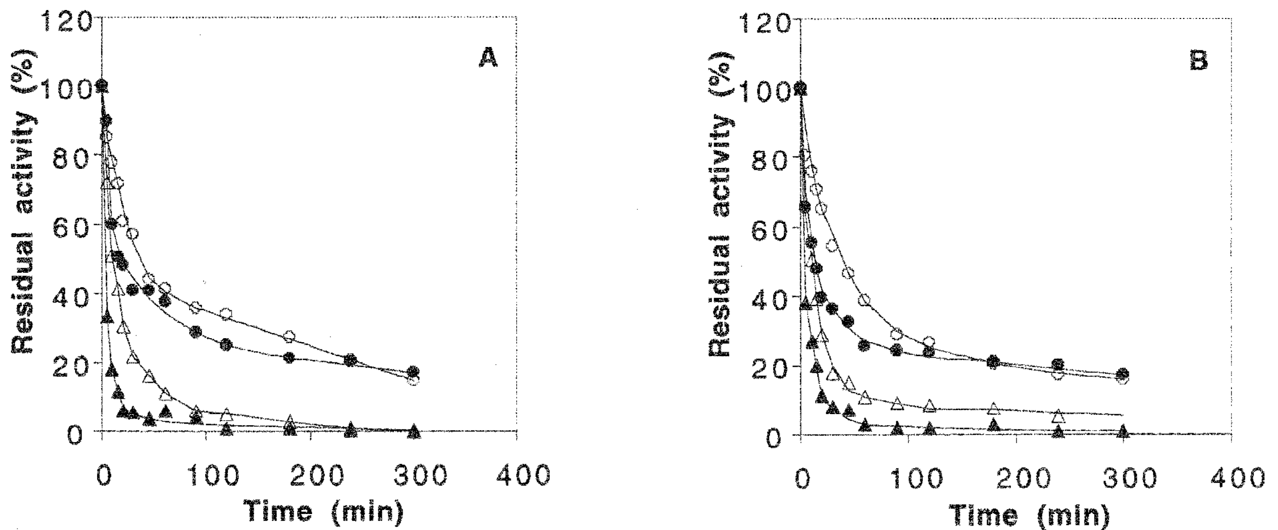

Figure 4. Effects of protein concentration on inactivation of -chymotrypsin at 50°C at pH 4 in citrate buffer and at pH 7 in phosphate buffer, with ATEE (A) and CPPNA (B) as substrates. Protein concentrations and pHs: (O) 0.1 mg/ml and pH 4, (●) 1 mg/ml and pH 4, ( ) 0.1 mg/ml and pH 7, (▲) 1 mg/ml and pH 7. For details, see text.

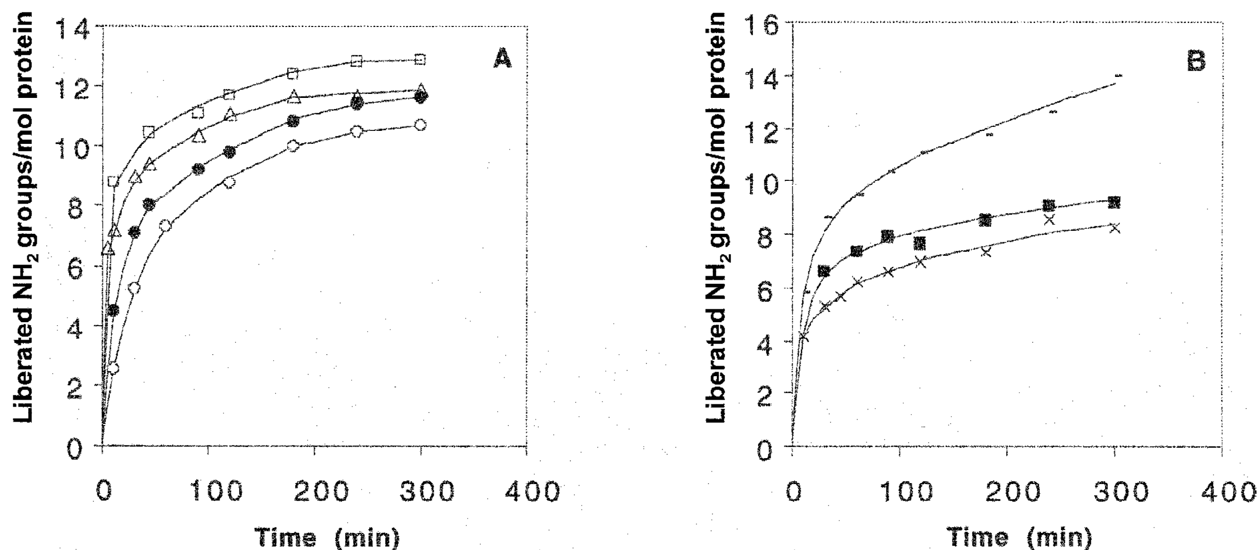

Figure 5. Effects of pH on autolysis of trypsin at 45°C. Protein concentration: 1 mg/ml. Buffers (0.1 M): citrate (O) pH 5, (●) pH 6; phosphate ( ) pH 7, (□) pH 8; Tris/HCl (–) pH 8; borate (■) pH 9, (x) pH 10. For details, see text.

The results obtained on the heat denaturation of - chymotrypsin at pH 6 and at 45°C point to consecutive reactions: the first step, heat denaturation, is followed by the digestion of the damaged molecules. Similar kinetics could not be observed for trypsin. We presume a higher sensitivity of trypsin for autodigestion, resulting in a very short, undetectable lag period.

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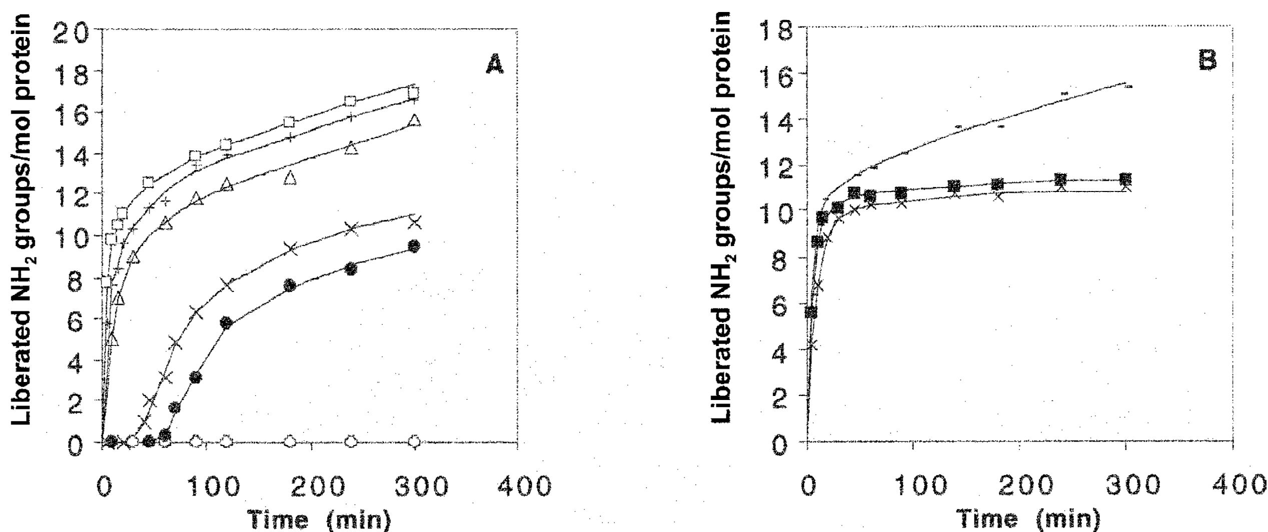

Figure 6. Effects of pH on autolysis of -chymotrypsin at 45°C. Protein concentration: 1 mg/ml. Buffers (0.1 M): citrate (O) pH 5, (●) pH 6; phosphate (X) pH 6, ( ) pH 7, (+) pH 7.5, (□) pH 8; Tris/HCl (–) pH 8; borate (■) pH 9, (x) pH 10. For details, see text.

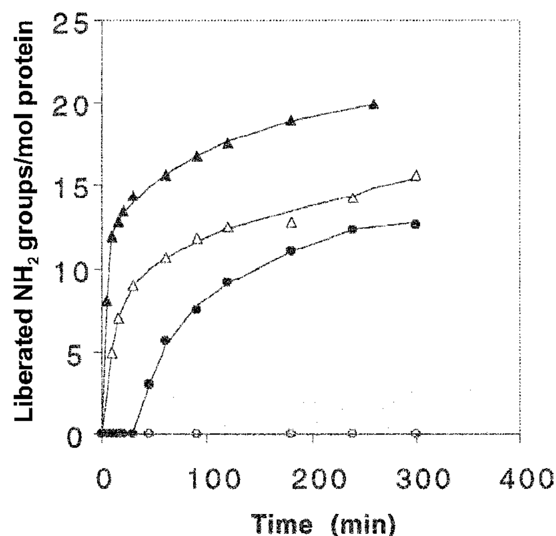

Figure 7. Effects of temperature on autolysis of -chymotrypsin Enzyme concentration: 1 mg/ml. Buffers (0.1 M) and temperatures: citrate (O) pH 4 and 4 °C, (●) pH 45 and 50°C, phosphate ( ) pH 7 and 45°C, (▲) pH 7 and 50 °C. For details, see text.

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