Salt Effects on Plastein Formation by α -Chymotrypsin

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The plastein formation by α -chymotrypsin from an ovalbumin hydrolysate was affected in an order of valency of salts when the concentration of each salt was 1 M. Monovalent cations were rather effective at this concentration and enhanced the plastein yield by 10%. In the presence of NaCl, the plastein formation showed two distinct maximal rates at its concentrations of 0.1 M and 0.8 M. The first maximum was considered to be resulted from an increase in enzyme activity, since chymotryptic hydrolysis of both *N*-acetyl-L-tyrosine ethyl ester and benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester was activated at an NaCl concentration of 0.1~0.2 M. The second maximum was ascribed to the salting-out of the product due to the higher concentration of NaCl. A salt-tolerant protease was also used to confirm the above conclusions. It was observed that this enzyme was much effective in producing a plastein at a high NaCl concentration. This may be due to the fact that both the enzyme activation effect and the product salting-out effect participate co-operatively.

A main mode of α -chymotrypsin in the plastein reaction has been proposed.^{1,2)} Several reports have been presented concerning the reaction conditions such as effects of substrate size,^{3,4)} substrate concentration,⁵⁾ and pH.⁶⁾ Besides these, a salt effect is considered to be important to study, especially from the standpoint of practical application of the plastein reaction. At least two effects are expectable from such a study. One is an effect of salt on enzyme activation or inactivation during the reaction and the other is its effect on salting-out of the product. If the latter factor is involved, the product is eliminated from the system by being salted out and accordingly the reverse reaction proceeds efficiently. This may be expected to have practical use in producing a plastein in a higher yield.

The present study investigates such salt effects to provide basic information to achieve the practical purpose mentioned above.

MATERIALS AND METHODS

Substrates. An ovalbumin preparation (20 g) from DIFCO was dissolved in 0.5 N NaOH (400 ml) and the solution was centrifuged at 5000 rpm for 20 min. The

supernatant was dialyzed through a cellophane membrane against running water for 2 days and against deionized water (10 liters) at 4°C for the following 2 days. This desalted ovalbumin solution was diluted to 2 liters with deionized water and incubated with 200 mg of subtilisin BPN' (Nagase Sangyo Co.) at 37°C for 24 hr. The hydrolysate was dialyzed through a cellophane membrane against deionized water (10 liters) at 4°C for 2 days to obtain a dialyzable fraction. This was freeze-dried and the resulting powder was used as a substrate for the plastein reaction. The following two amino acid derivatives were used for comparison: *N*-acetyl-L-tyrosine ethyl ester (ATEE) obtained from Kanto Kagaku Co. and carbobenzyloxy-L-phenylalanine *p*-nitrophenyl ester (Z-Phe-PNP) synthesized by the method of Bodanszky and Vigneaud.⁷⁾

Enzymes. α -Chymotrypsin (Miles Servac) and a salt-tolerant, neutral protease (tentatively named Neutral Protease II) from *Aspergillus sojae*⁸⁾ were used.

Determination of synthetic activity. A substrate solution was prepared by dissolving the ovalbumin hydrolysate (dialyzable fraction) in deionized water to a concentration of 35% (w/v). The pH was adjusted to 6.6. The enzyme-substrate ratio was set at 1/100 (w/w) and the reaction temperature at 37°C. In using Neutral Protease II the enzyme-substrate ratio was chosen as 1/3 (w/w). The reaction time was set at 2 hr because a linear time-response relation was obtained from a preliminary experiment. Furthermore, a relative activity V/V_0 was expressed as a ratio between a reaction rate in a given salt solution and that in dei-

onized water.

Determination of hydrolytic activity. Degree of hydrolysis of *N*-acetyl-L-tyrosine ethyl ester and carboxybenzyloxy-L-phenylalanine *p*-nitrophenyl ester were determined according to the decrease in the optical density at 237 nm⁹⁾ and to the increase in the optical density at 402 nm,¹⁰⁾ respectively. The relative activity V/V_0 was obtained similarly. More detailed description on the experimental procedures and the hydrolysis conditions is given in the legend to Fig. 2.

Determination of solubility. To an NaCl solution (1 ml) was added α -chymotrypsin and its solubility was determined by the turbidometric method described in a previous paper.³⁾ To an NaCl solution (5 ml) was added a freeze-dried plastein (25 mg) to determine its solubility similarly.

RESULTS

Effects of salts on plastein formation

When each of various salts was added to a reaction system in a concentration of 1 M, the plastein productivity was affected as the salt charge increased (Table I). In this case mono-monovalent salts, LiCl, NaCl and KCl, had a rather favorable effect and the plastein productivity was enhanced by about 10% in each case (Table I).

TABLE I. EFFECTS OF SALTS ON PLASTEIN PRODUCTIVITY OF α -CHYMOTRYPSIN

Salt	Ionic strength in the reaction system	Plastein productivity V/V_0
LiCl	1	1.12
NaCl	1	1.12
KCl	1	1.13
MgCl ₂	3	0.80
CaCl ₂	3	0.65
CoCl ₂	3	0.15
ZnCl ₂	3	0.15
AlCl ₃	6	0.17
FeCl ₃	6	0.05
NaNO ₃	1	1.01
NaNO ₂	1	1.01
CH ₃ COONa ^{a)}	1	0.75
Na ₂ SO ₄	3	1.01
Na ₂ SO ₃	3	0.86
Na ₃ PO ₄ ^{a)}	6	0.81

^{a)} In each reaction pH was adjusted to 6.6 with NaOH or HCl.

The dependence of the plastein produc-

tivity on the salt concentration was investigated with NaCl and the plastein productivity plotted as a function of ionic strength showed two maxima at 0.1 and at 0.8 (Fig. 1).

FIG. 1. Plastein Productivity as a Function of Ionic Strength of NaCl.

Effect of NaCl upon the hydrolytic activity of α -chymotrypsin

α -Chymotryptic hydrolysis of ATEE was investigated under a usual condition and of Z-Phe-PNP under such a condition as approximated to that for the plastein reaction. The ATEE hydrolyzability showed a maximum at ionic strength of 0.3. There was a maximum at ionic strength of 0.2 in hydrolysis of Z-Phe-PNP. In both cases the hydrolytic activities of α -chymotrypsin decreased as this enzyme was salted-out by NaCl (Fig. 2).

Salting-out effect of NaCl upon plastein

Solubilities of a plastein at various NaCl concentrations are shown in Fig. 3. From this result it is estimated that the plastein is salted-in at ionic strength of about 0.5 and salted-out above this ionic strength.

NaCl effect on plastein formation by a salt-tolerant protease

Neutral Protease II whose activity was not affected by salt was used to produce a plastein at various NaCl concentrations and this study

FIG. 2. Effects of NaCl upon the Hydrolytic Activity of α -Chymotrypsin toward *N*-Acetyl-L-tyrosine Ethyl Ester (○—○) and toward *N*-Benzoyloxycarbonyl-L-phenylalanine *p*-Nitrophenyl Ester (●—●) and upon the Insolubilization of this Enzyme (○---○).

To 0.01 M phosphate (pH 7.8) containing 5% CH_3CN and a given concentration of NaCl, was dissolved ATEE to a concentration of 2 mM. α -Chymotrypsin was dissolved in deionized water to a concentration of 5×10^{-7} M. A 1:10 mixture of both solutions was incubated at 18°C for 4 min and the ATEE hydrolysis followed by measuring a decrease in optical density at 237 nm.

The desalted protein hydrolysate (350 mg) was dissolved in deionized water (0.9 ml) containing 5% CH_3CN and a given concentration of NaCl. To this solution was dissolved Z-Phe-PNP to a concentration of 0.2 mM. α -Chymotrypsin (0.1 mg) was dissolved in deionized water (1 ml). A 1:9 mixture of both solutions was incubated at 18°C for 15 min. The reaction was stopped by adding 4 ml of 0.1 N HCl. The liberated PNP was extracted with 10 ml of CH_2Cl_2 -ether (4:1). After evaporating the solvent, the residue was dissolved in 2.0 ml of 0.1 M phosphate (pH 7.8) and the optical density measured at 402 nm.

α -Chymotrypsin (5 mg) was added to an NaCl solution (1 ml) having a given ionic strength and the resulting turbidity was measured at 600 nm.

gave a result shown in Fig. 4. The productivity increased with the NaCl concentration and doubled at 3 M.

DISCUSSION

Chymotryptic plastein formation was changeable in accordance with the lyotropic series of salts. Mono-monovalent salts generally enhanced the plastein formation (Table I). Therefore we picked out NaCl as a represen-

FIG. 3. Effect of NaCl on Solubility of a Plastein.

A plastein (25 mg) was added to an NaCl solution (5 ml) having a given ionic strength and the resulting turbidity was measured at 600 nm.

FIG. 4. Effect of NaCl on the Plastein Formation by a Salt-tolerant Protease.

tative and investigated the plastein formation as a function of NaCl concentration. We observed the two maximal plastein yields at ionic strength 0.1 and 0.8 (Fig. 1). In order to make clear this phenomenon, the NaCl effect on hydrolytic activity of the enzyme was investigated. As shown in Fig. 2, only one maximum was observed at ionic strength 0.2. This fact suggests that the first maximum results from an increase in the enzyme activity. Similar phenomena have been reported in the case of carboxypeptidase A¹¹⁾ and glutamate dehydrogenase.¹²⁾

The second maximum was observed at a higher ionic strength region where many

proteins were easily salted-out.¹³⁾ The plastein reaction product was salted-in below an ionic strength of about 0.5 and salted-out above this ionic strength (Fig. 3). But the salting-out may happen at lower ionic strength in plastein synthesis by reason the concentration of substrate was 70 times higher as compared with this experimental condition. From this result the second maximum is ascribable to the salting-out of the product due to the higher concentration of NaCl.

A salt-tolerant proteinase from *Aspergillus sojae* (neutral protease II) was also used to obtain a further positive proof. As expected, a higher NaCl concentration was much more favorable in plastein synthesis than hydrolysis (Fig. 4). This may have practical use in producing a plastein more efficiently by using salt tolerant enzymes.

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