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Enzyme-catalyzed peptide syntheses in aqueous polymer two-phase systems

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Summary

Peptide synthesis in the aqueous polymer two-phase system was examined. Water soluble polymers adopted were dextran and polyethyleneglycol (PEG). By the modification of a proteolytic enzyme, trypsin, with dextran carrying a very small number of carboxyl groups, a separation of the enzyme from its peptide product, N-benzoyl-L-arginine glycinamide, which is relatively more soluble in a PEG phase could be realized. This separation drastically reduced a so-called "mass-law" effect, and increased the yield of the reaction product. The aqueous polymer two-phase system would be useful to enhance the usefulness of biocatalysts in organic syntheses.

Introduction

Water soluble polymers such as dextran and polyethyleneglycol (PEG) are known to make a phase separation in water. This phenomenon is attributed to the repulsion between monomer units of the polymers; that is, when two kinds of polymers coexist, a mixing entropy of the system is much smaller than that of the system where two kinds of monomeric units independently exist, which induces a liquid-liquid phase separation even if the repulsive force between two kinds of monomer units in the polymers is small (1,2).

By the modification of an enzyme with one of the pair of polymers which show the liquid-liquid phase separation phenomenon, we can expect a separation of the enzyme from its product, if the reaction product of the enzyme is relatively more soluble in a polymer solution of the other side. This separation might drastically reduce a so-called "mass-law" effect which decelerates the enzyme reaction (3). This is a basic principle of the present study. To realize this idea, we examined peptide synthesis by trypsin in the polymer two-phase system.

Experimental

Materials

Dextran (Mw = 170,000) and polyethyleneglycol (Mw = 50,000) were purchased from Nacalai Tesque, Kyoto, Japan, and Wako Pure Chemicals, Osaka, Japan, respectively. Trypsin (bovine pancreas) was from Sigma, St. Louis, Mo. Other reagents were commercially available. A Milli-Q grade water was used to prepare sample solutions.

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Carboxymethylation of dextran

Dextran was reacted with 2-chloroacetic acid in a NaOH solution (Table 1), and the reaction mixture was dialyzed against water for a week. After neutralization by stirring with ion-exchange resins (Amberlite CG-120 Type 1, H^+-form) and subsequent filtration, the carboxymethylated dextran (MDX) obtained was lyophilized. The number of carboxyl groups introduced to the dextran molecule was estimated by the conductometric titration with a N/100 NaOH solution.

Modification of trypsin with carboxymethyl dextran

Trypsin was incubated with carboxymethyl dextran in the presence of water soluble carbodiimide (WSC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, at pH 5.0 and at 5 °C for 21 h. To protect the active site of the enzyme, N-benzoyl-*L*-arginine hydrochloride (5 mM) was added in the coupling solution. The solution mixture was ultrafiltrated (Amicon Model 8010, membrane XM-50, exclusion limit 50,000) until the filtrate showed no absorbance at 280 nm (Ubest 35 UV-visible spectrophotometer, Japan Spectroscopic Co., Tokyo, Japan).

Measurements of catalytic activity of enzymes

Catalytic activity of free and modified trypsins in the hydrolyses of N-benzoyl-*L*-arginine ethyl ester hydrochloride (BAEE, 0.33 mM) and N-benzoyl-*D,L*-arginine *p*-nitroanilide (BANA, 0.33 mM) was followed spectrophotometrically (wavelengths followed; 247 nm (BAEE) and 410 nm (BANA)).

Peptide synthesis by trypsins

N-Benzoyl-*L*-arginine ethyl ester hydrochloride (BAEE, 0.069 g) and glycinamide (Gly-NH_2, 0.022 g) were dissolved in a Tris buffer (0.25 M, pH 10, 0.98 ml. 20 mM of $CaCl_2$ was added) in a small test tube. In the polymer-two phase system, PEG (4.65 wt%) and dextran (4.65 wt%) were added. Finally a solution of enzyme was added and the solution mixture was quickly stirred at 25 °C.

In the catalysis by trypsin modified with dextran, the modified enzyme was stirred (700 rpm) with a solution mixture of BAEE, Gly-NH_2, dextran (without enzyme) and polyethyleneglycol, while the total amount of dextran in the test tube was kept constant. A small aliquot (0.1 ml) was pipetted out at appropriate intervals, and the conversion of the product, N-benzoyl-*L*-arginine glycinamide, was estimated from the amount of unreacted glycinamide by using the ninhydrin method at 570 nm (4), because only glycinamide has a free α-amino group.

Table 1. Introduction of Carboxymethyl Groups into Dextran[a]

Dextran	NaOH (mg)	Cl-CH_2-COOH (mg)	H_2O[b] (ml)	Time (h)	Temp (°C)	DS[c]
MDX1	4.64	10.98	7.2	1	5	2.2
MDX2	4.64	10.98	7.2	6	5	20.6
MDX3	0.46	1.10	7.0	1	5	1.8
MDX4	0.46	1.10	7.0	1	25	2.1

a. 2 g of dextran was used. b. total volume

c. Number of carboxyl groups in a dextran molecule

Phase separation experiment

Two kinds of polymers were dissolved in the Tris buffer in a small test tube, and left for 15 min at 25 °C in a water bath. The appearance of boundary layer in the solution mixture was checked by naked eyes.

Results and discussion

A. Catalytic activity of trypsins

From the absorbance of the filtrate at 280 nm after the ultrafiltration, the number of trypsin covalently bound to the carboxymethyl dextran molecule (MDX-1) was estimated to be 0.9 (Table 2). The trypsin modified with dextran (MDX-Ea) had 49 % and 59 % catalytic activities relative to those of free enzyme in the hydrolyses of BAEE and BANA, respectively (Table 3).

It was confirmed that the presence of neither free dextran nor PEG showed any influence on the rate of hydrolysis of BAEE by free trypsin in the concentration ranges examined here.

B. Peptide synthesis

A mixture of dextran and PEG showed a phase separation in a Tris buffer (Figure 1 (a)). The carboxymethyl dextran made a phase separation with PEG in a more condensed region than the non-modified dextran (Figure 1 (b)) due to the presence of carboxymethyl groups which slightly disturb the phase separation. By the partial replacement (17 %) of MDX by trypsin-carrying carboxymethyldextran, however, the phase separation phenomenon was not disturbed (Figure 1 (c)).

By stirring the mixture of carboxymethyldextran and PEG at 700 rpm the solution became turbid, which showed the retainment of phase separation between two polymers. In this aqueous polymer two-phase system, the trypsin-catalyzed synthesis of N-benzoyl-*L*-arginyl-glycinamide (Bz-*L*-Arg-Gly-NH_2) from N-benzoyl-*L*-arginine ethyl ester (BAEE) and glycinamide (Gly-NH_2) was examined (3,4,5). When trypsin is incubated with the specific ester substrate, BAEE, in the presence of Gly-NH_2, the reaction intermediate, acylated trypsin, is attacked by Gly-NH_2 or water, which results in the objective amide (Bz-*L*-Arg-Gly-NH_2), or N-benzoyl-*L*-arginine, respectively (Scheme I).

Table 2. Modification of Trypsin with Carboxymethyl Dextran

Trypsin	MDX (g)	[E][a] (mg/ml)	[MDX][a] (mg/ml)	DS[b]
MDX-Ea	MDX1 1.0	6.25	51.50	0.9
MDX-Eb	MDX2 0.1	8.49	10.81	5.8

a. Concentration after the purification procedures
b. Number of enzyme molecules introduced into a carboxymethyl dextran molecule

Table 3. Catalytic Activity of Native and Modified Trypsins[a]

Trypsin	Esterase Activity (mM/s)	Amidase Activity (nM/s)
Native	0.53	13.5
MDX-Ea	0.26	7.9

a. $[E]_o$ = 4.5 mM, $[S]_o$ = 0.33 mM.

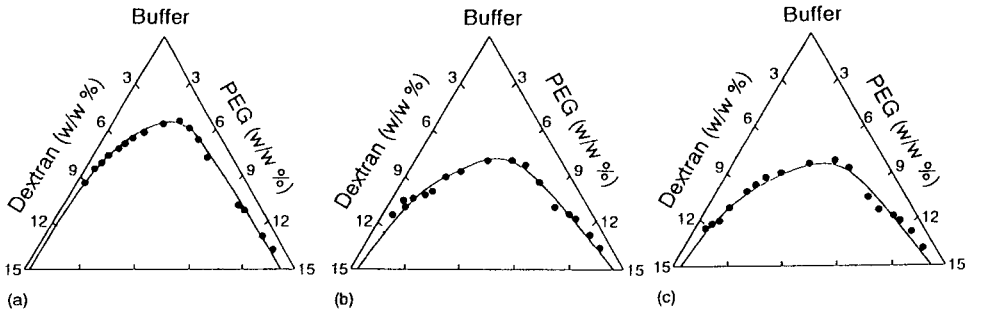
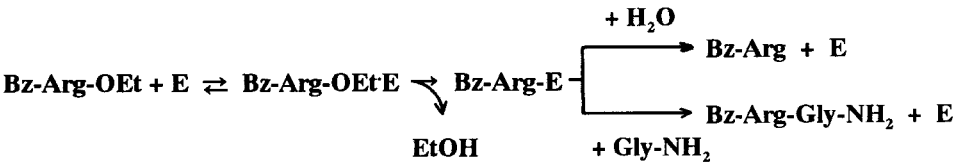


Figure 1. Phase diagrams for a mixture of PEG and dextran derivatives at 25 °C in a Tris buffer (pH 10, 0.25 M). (a) PEG / Dextran (b) PEG / Carboxymethyldextran (MDX-1, DS=2.2). (c) PEG / Trypsin-carrying carboxymethyldextran (MDX-Ea) - MDX-1 mixture (MDX-Ea : MDX = 17 : 83).



Sheme I Catalytic Process of Trypsin

Figure 2 shows time evolutions of the amount of Bz-*L*-Arg-Gly-NH_2 in the presence of (a) free trypsin in homogeneous solution, (b) free trypsin in the two-phase, and (c) modified trypsin in the two-phase. Only in catalysis by the modified enzyme in the two-phase system, the yield of the product remained 30-40 % for several ten minutes. In other systems, the yield rapidly decreased after passing the maximum due to the trypsin-catalyzed hydrolysis of the product Bz-*L*-Arg-Gly-NH_2 (5). These results clearly show usefulness of the aqueous polymer two phase system examined in this work.

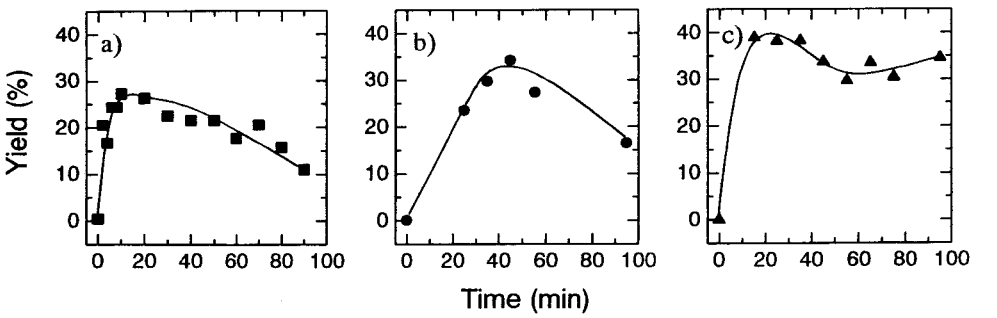


Figure 2. Time evolutions of the yield of Bz-*L*-Arg-Gly-NH_2 catalyzed by (a)free trypsin in homogeneous solution, (b) free trypsin in two-phase system, and (c)trypsin modified with carboxymethyl dextran in two-phase system. Tris buffer (0.25 M, pH 10.0), [$CaCl_2$] = 20 mM, [BAEE] = 0.2 M, [Gly-NH_2] = 0.2 M, [E] = 50 mM, 25 °C.

Table 4. Distribution Ratios of Various Components in Polymer Two-Phase System[a]

Component	Lower Phase (Dextran) / Upper Phase (PEG)
Free Trypsin	0.94
Dextran-Trypsin	2.15
Gly-NH_2	1.38
Bz-Arg-OEt	0.98
Bz-Arg-Gly-NH_2	0.95

a. concentration ratio. The volume ratio (PEG phase / dextran phase) was 3.3.

C. Distribution of reactants and product in the polymer two-phase system

To interpret the results in Figure 2, a distribution of components including enzymes was evaluated in the two-phase system (Table 4). Free trypsin was preferentially distributed in PEG phase. As for the trypsin bound to dextran, on the contrary, the enzyme was more preferentially distributed in the dextran phase than in the PEG phase (2.15 : 1). It should be mentioned here that the appearance of aqueous polymer two-phase system does not mean that two kinds of polymers exist completely separately. Even in the PEG phase, some amount of dextran is dissolved, and vice versa. As for BAEE, its distribution in dextran phase was approximately the same as that in the PEG phase, whereas that of glycinamide in the dextran phase was larger than that in the PEG phase.

The distribution of the reaction product, Bz-*L*-Arg-Gly-NH_2, was checked by uv measurements, and found that the product was slightly more preferentially distributed in the PEG phase, which induced the effective production of Bz-*L*-Arg-Gly-NH_2 by the modified enzyme and Gly-NH_2 preferentially located in the dextran phase, and subsequent transport of the product to the PEG phase.

In conclusion, the aqueous polymer two-phase system examined here is effective to separate the enzyme modified with dextran from the reaction product, which results in a larger conversion of the reaction product.

Acknowledgment

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