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Complexation of pepsin poly(ethylene glycol)

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Summary

Complexation of porcine pepsin (or pepsin A) (EC.3.4.23.1) with poly(ethylene glycol) (PEG) in an aqueous solution was studied as a function of pH and PEG concentration. The addition of PEG increased the reduced viscosity of the enzyme solution at pH 3 but not at pH 4.5. An increase in pH was observed by mixing both PEG and enzyme solutions which were previously adjusted to pH 3. Under conditions of pH 2.5–3 and 50°C, PEG contributed to an increase in the hydrolyzing activity of pepsin toward *N*-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine. These results indicate that pepsin forms a water-soluble complex with PEG mainly through hydrogen bonds between the carboxyl groups in the enzyme and the ether groups in PEG.

Introduction

The complexation of proteins with water-soluble synthetic polymers in an aqueous system is interesting from the following two points of view. The first concerns how the polymers interact with non-flexible protein molecules through electrostatic force, hydrogen bonding and hydrophobic interaction; the second is to what extent biochemical ability is maintained in the resulting complexes. Previously we studied the complexation of strongly acidic and basic polyions with human carboxyhemoglobin (1,2), human serum albumin (3) and bovine trypsin (4). It has become apparent that in spite of the combination of protein and polyelectrolyte, complexation took place through stoichiometric salt-linkages between oppositely charged groups to result in a water-insoluble complex. There was an appreciable retention of biochemical functions in the resulting stoichiometric complexes (4,5). Thus, a study of the complexation of proteins with water-soluble nonionic polymers becomes of interest.

Porcine pepsin (pepsin A) is well known as a typical acid gastric protease including a number of carboxyl groups. This enzyme has already been a topic of research with regard to obtaining information about amino acid sequence, three-dimensional conformation, enzymatic properties and so on (6). We thus attempted to investigate the complexation of pepsin with poly(ethylene glycol) (PEG) in an aqueous system. PEG is suitable for the present purpose

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because it is capable of forming a complex through hydrogen bonding with the carboxyl groups in a polymer such as poly(acrylic acid) (7) or poly(methacrylic acid) (8).

Materials and Methods

Pepsin (from porcine stomach mucosa) was commercially obtained from Sigma Chemical Co. Its nominal enzyme activity was 3500±300 units/mg protein when one unit was defined as having an absorbance change of 0.001 per minute at 280 nm during digestion of hemoglobin at pH 2.0 and 37°C. The molecular weight of pepsin was 34542, as determined by the amino acid sequence. The enzyme sample was used after its purity was confirmed by amino acid analysis and electrophoresis in polyacrylamide gel. PEG ($\overline{Mn}$ = 1.98X10^4) was the same as the sample used in the previous study (9). *N*-Acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine (APDT) was purchased from Sigma Chemical Co. All other reagents were of analytical grade and obtained from commercial sources.

The complexation was studied through a combination of viscometric measurements, pH measurements and circular dichroism (CD) measurements. Viscosity was measured at 25±0.005°C for aqueous solutions (pH 3.0 and 4.5) containing 0.1 g/dl of the enzyme and different amounts of PEG, using an Ubbelohde viscometer with a follow time of 304.5 sec for water at 25°C. To examine a change in the proton concentration during the complexation, aqueous pepsin and PEG solutions previously adjusted to the same pH, were mixed and subjected to pH measurement which was performed at 25±0.1°C with a Beckman model 4500 pH meter. CD spectra were measured with a Jasco model J-40C spectropolarimeter.

The APDT-hydrolyzing activity was determined under conditions of temperature 25–60°C and pH 1.5–5.0 using aqueous sample solutions including 9.1 μg/ml of pepsin and different amounts of PEG. The resulting diiodo-L-tyrosine was analyzed by measuring color changes after its reaction with ninhydrin and also confirmed by amino acid analysis.

Results and Discussion

The amino acid sequence of porcine pepsin has previously been studied (10,11), thus the number of the ionizable groups can be counted: 59 total acidic groups (43 carboxyl, 16 phenolic OH, and no mercapto free from disulfide linkages); 5 total basic groups (2 amino, 1 imidazolyl, and 2 guanidyl). Of these ionizable groups, both COOH and phenolic OH are expected to take part in the complexation with PEG through hydrogen bond formation. The complexation of pepsin with PEG was studied under various levels of pH and molar ratio (R_m) of the ether groups in PEG to all pepsin acidic groups. In contrast to polyelectrolyte-protein systems (1-4), PEG and pepsin did not form a precipitated water-insoluble complex under the conditions used here, *i.e.*, R_m = 0.1 to 150 and pH = 1 to 5. Thus, at first we adopted the viscometric method; the reduced viscosity (η_{sp}/C_{pepsin}) of aqueous pepsin solutions including different amounts of PEG was examined at pH 3.0 and 4.5 (Fig. 1). Because

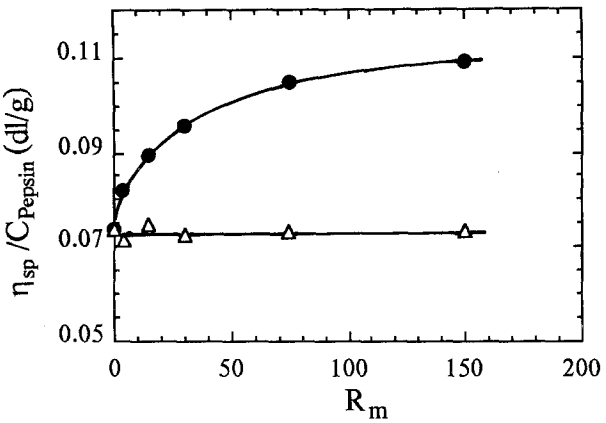


Fig. 1. Change in reduced viscosity of aqueous pepsin solution with R_m at pH 3.0 (●) and 4.5 (Δ). The enzyme concentration was kept constant (0.1 g/dl) through all the measurements.

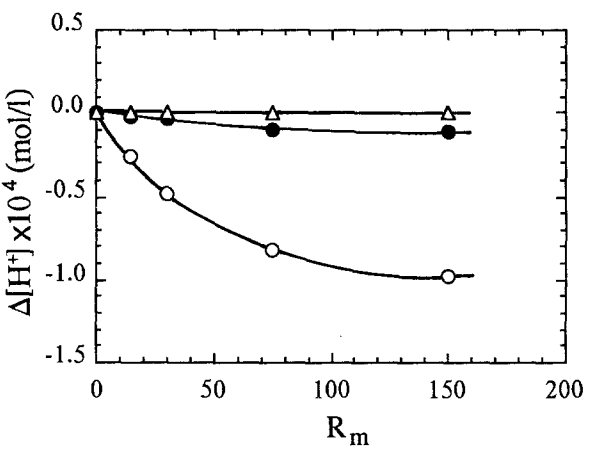
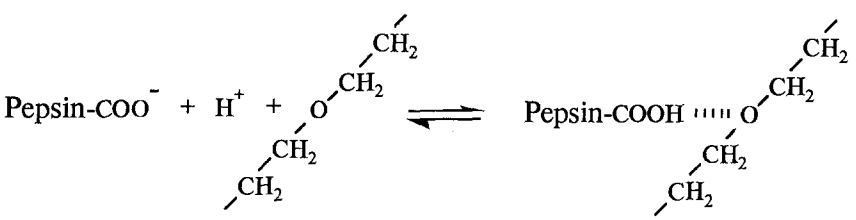


Fig. 2. Difference in the proton concentrations of pepsin solutions containing and not containing PEG. The initial pH values of both the enzyme and PEG solutions were adjusted to 3.03 (○), 4.36 (●), and 6.82 (Δ) before mixing. All of the sample solutions contained a constant concentration (0.1 g/dl) of the enzyme. The amount of the proton uptake by one mole of pepsin at pH 3.03 and Rm = 150 was about 8.2% of all the pepsin COOH groups, as calculated from the data obtained.

of $\eta_{sp}/C_{pepsin} = \{(\eta/\eta_o) - 1\}/C_{pepsin}$, where η is the viscosity of the sample containing both pepsin and PEG and η_o is the viscosity of the solvent only including PEG, the reduced viscosity remains constant when the pepsin concentration (C_{pepsin}) is unchanged and also when the enzyme does not interact with PEG to change the η/η_o ratio. As can be seen from **Fig. 1**, the value of η_{sp}/C_{pepsin} at pH 4.5 was independent of R_m, indicating no interaction between pepsin and PEG. In contrast, the viscosity levels at pH 3.0 increased in response to higher Rm levels. This reveals that at pH 3.0, PEG interacts with pepsin to form a water-soluble complex having a large molecular weight relative to the free enzyme. In addition, of the pepsin acidic groups only COOH appears to take part in the complexation. This is because the viscosity varied at pH 3.0, but not at pH 4.5 where the phenolic OH groups in polypeptides and proteins are undissociated (12).

The complexation was further studied by monitoring pH changes with the addition of PEG into the enzyme solution. **Figure 2** shows differences in the proton concentrations ($\Delta[H^+]$) before and after the mixing of the enzyme and PEG solutions. Initially, both of these two solutions were adjusted to the same pH value. In the range of pH ≥ 4.36, $\Delta[H^+]$ is almost independent of R_m. Taking into account the results of the viscometric measurements, it is likely that in this pH range PEG does not form a complex with pepsin. However, when the initial pH was adjusted to 3.03, $\Delta[H^+]$ decreased with increasing R_m and the pattern of the curve was analogous to the viscosity curve obtained at pH 3.0. This indicates that the complexation of PEG with pepsin was accompanied by proton uptake (*i.e.*, an increase in pH). Since it has been suggested that the carboxyl groups in pepsin are responsible for the complexation (see above), the proton uptake seems to occur by the hydrogen bonding of COOH with the ether groups in PEG:



It is interesting to study how the hydrogen bond formation affects the three-dimensional structure of pepsin. The CD spectra were measured under the same conditions as used in the viscosity measurements. There was no distinguishable difference in the spectral data before and after the complexation. Therefore, pepsin molecules appear to loosely associate with PEG through hydrogen bonding without a conformation change in the enzyme.

Another important subject of this study is to clarify to what extent enzymatic activity is maintained in the pepsin-PEG complex. The hydrolyzing activity of pepsin toward APDT was examined in aqueous systems containing different amounts of PEG as a function of pH and

temperature (**Table 1**). Relative activity was then calculated from the initial rate of APDT hydrolysis by the native and complexed enzymes under various conditions. When the activity was assayed at pH 3 and 50°C, the added PEG obviously increased the enzymatic activity of pepsin (see No. 1–5 in **Table 1**). This is due to the resistant effect of PEG on the decrease in activity of pepsin with an increase in pH (see No. 10–16). The active center of pepsin is known to consist of several amino acid residues, including two aspartic acids – positions 32 and 215 in the amino acid sequence as reported by Sepulveda *et al* (11). The dissociation constants (*pKa*) of each COOH group in both aspartyl residues are 1.2 (Asp-32) (ref.13) and 4.5 (Asp-215) (ref.14). Therefore, the PEG-induced activity change can be explained as follows: PEG forms the hydrogen bonds with a part of the pepsin COOH groups which are exposed to the aqueous phase and thus influences the ionization state of the enzyme molecules to alter the *pKa* values of Asp-32 and/or Asp-215. This possibility can be supported by the fact that proton uptake was observed during the complexation (see **Fig. 2**).

TABLE 1
Enzymatic Activity of Native and Complexed Pepsin[a,b,c]

No.	R_m	pH	Temp (°C)	Relative Activity (%)	
				Native[d]	Complexed
(Effect of R_m)					
1	0	3.0	50	82.9	
2	15	3.0	50		98.2
3	30	3.0	50		102
4	75	3.0	50		100
5	150	3.0	50		99.9
(Effect of temperature)					
6	30	3.0	20	16.5	16.4
7	30	3.0	37	55.3	55.6
8	30	3.0	50	83.7	100
9	30	3.0	65	33.3	23.2
(Effect of pH)					
10	30	1.5	50	47.9	50.9
11	30	2.0	50	100	100
12	30	2.5	50	98.2	101
13	30	3.0	50	83.3	100
14	30	3.5	50	41.6	43.5
15	30	4.0	50	23.4	23.4
16	30	5.0	50	8.6	8.6

[a] 100% activity refers to the hydrolyzing rate by the native enzyme at pH 2.0 and 50°C. [b] The results in No. 1, 3, 8 and 13 were obtained from different runs of the measurements. [c] The range in the measured relative activities averages ±0.9% with none being greater than ±2.3%. [d] No. 6 to 16, as well as No. 1, show the data at R_m = 0.

In addition to these results, complexed pepsin was found to be inferior to the native enzyme in regard to activity at 65°C (No. 9 in **Table 1**), but at 50°C the level of activity was reversed (No. 8). Such a temperature effect can be understood by assuming a hydrophobic interaction between PEG and pepsin. This is consistent with the well known fact that the hydrophobic interaction is strengthened by a rise in temperature. For example, in the low temperature range there is no marked difference in the activities between the native and complexed enzymes (No. 6 and 7). Based on these experimental results we can say that the hydrohobic interaction plays, at least in a high temperature range, a role in the complexation of PEG with pepsin.

In conclusion, it has become apparent that porcine pepsin as a typical acidic protease loosely associates with PEG and forms a water-soluble complex in the aqueous system under acidic conditions at pH ≤ 3. The complexation is mainly based upon the hydrogen bonding between the carboxyl groups in pepsin and the ether groups in PEG. This hydrogen bond formation resists a pH-induced activity fall. In order to understand the enzymatic property of the complex at a high temperature range, however, it is also necessary to take into account the hydrophobic interaction between pepsin and PEG.

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