Insolubilizing Studies of Water-Soluble Poly(Lys Tyr) by Tyrosinase[1)]

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Synopsis. Insolubilization of water-soluble synthetic poly(Lys Tyr) was examined using tyrosinase in water and simulated seawater systems. Tyrosinase oxidized tyrosine aromatic nuclei, causing intermolecular crosslinking reactions. The salinity, system pH and α-chymotrypsin are considered to play roles in the insolubilizing reaction.

It has been widely observed in both sea and fresh water that water-soluble proteins normally remain insoluble or surface adhesive in biological systems. Lindner et al.[2)] proposed a mechanism called auto-crosslinking to explain the adhesive properties of proteins secreted by barnacles and mussels. Auto-crosslinking occurs between tyrosyl residues of protein side-chains and the free amino or other reactive groups of other protein molecules with a phenolase enzyme forming an intermolecular crosslink. In this mechanism an oxidase enzyme secreted from byssus and enzyme glands of *Mytilus edulis* plays an initial primary role in protein insolubilization, followed by adhesion.[3,4)] Oxidase also participates in arthropods and chicken-egg shell hardening on land.[5)] These adhesive and hardening phenomena, however, consist of many complex factors, and the auto-crosslinking mechanism described above has not yet been confirmed in a simple protein model.

We have investigated the polymer chemistry of some polypeptide models of *Mytilus edulis*, barnacle, silkworm and caddisworm.[6–9)] This report describes insolubilizing experiments on synthetic poly(Lys Tyr) using tyrosinase in the presence of activator α-chymotrypsin.

Experimental

Poly(Lys[1] Tyr[1]) (hydrobromide, molecular weight 90000), tyrosinase and α-chymotrypsin were purchased from Sigma. Simulated sea water was produced according to the analytical values of six major inorganic salts (NaCl, $MgCl_2$, Na_2SO_4, $CaCl_2$, KCl, and $NaHCO_3$).[10)] The viscosity of poly(Lys Tyr) with and without an enzyme was measured using an Ubbelohde viscometer at 25 °C in both fresh water and seawater. The absorption spectra was measured using a spectrophotometer (Jasco UVIDEC-1).

Results and Discussion

Poly(Lys Tyr) is soluble in fresh water but not in real and simulated seawater. It can, however, be solubilized when the salt concentration is less than 20% of real seawater, with a pH of less than 9. The viscometric characteristics of poly(Lys Tyr) in water and simulated seawater (20% concentration) with and without tyrosinase are depicted in Fig. 1. A slight decrease in the specific viscosity of the polypeptide was found in water. In 20% seawater poly(Lys Tyr), with and without tyrosinase, increases its viscosity to similar levels within 20 h to a 4-fold maximum after 2 days. This increase is attributable to a hydrophobic stacking of tyrosine aromatic nuclei in the polypeptide side chains. After 20 h the poly(Lys Tyr) solution with tyrosinase exhibited a pale brownish purple color. In the tyrosinase free system, however, a white precipitate developed after 70 h. When α-chymotrypsin is added to a poly(Lys Tyr) solution containing tyrosinase, the

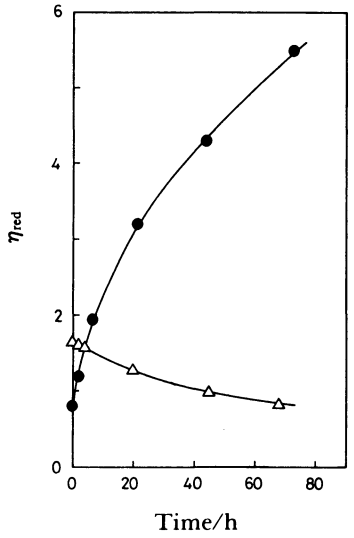

Fig. 1. Apparent viscosity change of poly(Lys Tyr) in aqueous media at pH 6.5 and 25 °C: △ with and without tyrosinase in water; ● with and without tyrosinase in 20% simulated seawater. Concentrations: poly(Lys Tyr) 0.25%; tyrosinase 1200 units.

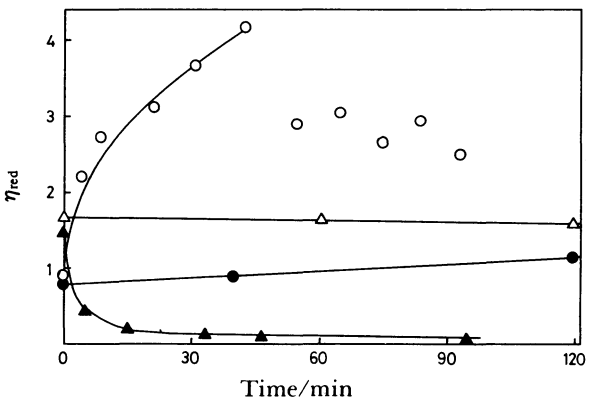

Fig. 2. Viscosity change of poly(Lys Tyr) in aqueous media at pH 6.5 and 25 °C: ○ tyrosinase and α-chymotrypsin in simulated seawater; ▲ tyrosinase and α-chymotrypsin in water; ● both with and without tyrosinase in simulated seawater; △ both with and without tyrosinase in water. Concentrations: poly(Lys Tyr) and tyrosinase, see caption for Fig. 1; α-chymotrypsin 3.6 units; 20% simulated seawater.

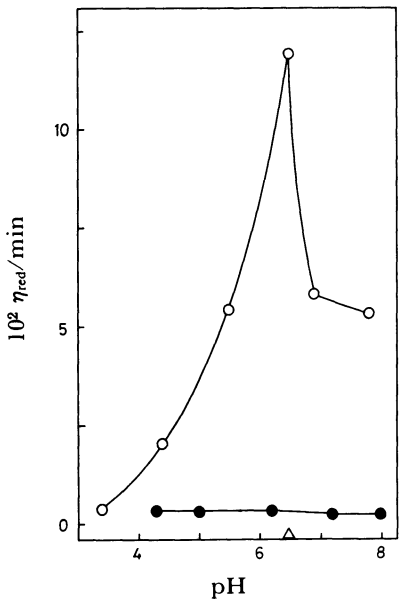

Fig. 3. pH dependence of viscosity change of poly-(Lys Tyr) in aqueous media at 25 °C: ○ tyrosinase and α-chymotrypsin in simulated seawater; ● tyrosinase in simulated seawater; △ tyrosinase in water. Concentrations, see captions for Figs. 1 and 2.

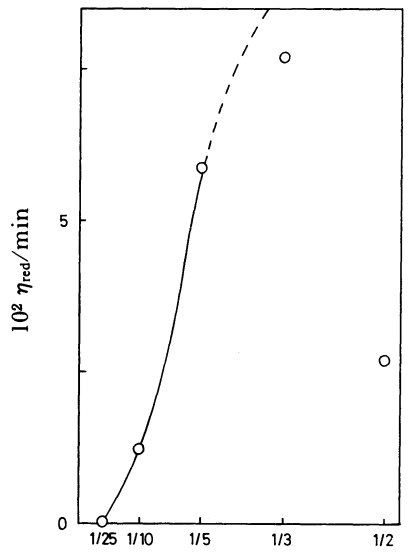

Fig. 4. Effect of salinity on viscosity change of poly(Lys Tyr) in simulated seawater. --- Turbidity observed in freshly prepared solution.

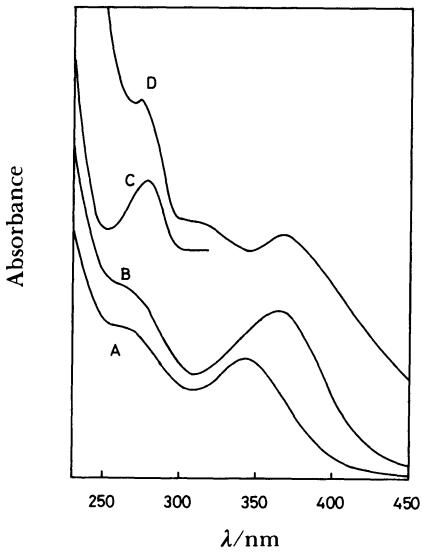

Fig. 5. Absorption spectra of products of poly(Lys Tyr) and model compounds. (A) Boc-L-Dopa quinone and (B) crosslinked Boc-L-Dopa quinone-α-Boc-L-lysine in water–alcohol (1:1 v/v);(C) poly(Lys Tyr) in simulated seawater; (D) 8 mol dm^{-3} HCl solution of partially hydrolyzates from the reaction of poly(Lys, Tyr), tyrosinase and α-chymotrypsin.

viscosity increases rapidly for up to 45 min and a precipitate may be observed by naked eye after 50 min in 20% sea water; the viscosity thereafter gradually decreases to 100 min (Fig. 2). The complex results shown in Figs. 1 and 2 suggest that a system containing poly(Lys Tyr), tyrosinase, and α-chymotrypsin in seawater acts as an auto-crosslinking model. α-Chymotrypsin is believed to cleave the polypeptide at its Tyr sites and to accelerate oxidation at the polypeptide terminals by tyrosinase, followed by the formation of intermolecular crosslinks.

The effects of pH on the viscosity change are depicted in Fig. 3. The viscosity of poly(Lys Tyr) in the presence of tyrosinase slowly decreased in water (pH 6.5) over 4 days. Tyrosinase in seawater induces a minor pH independent of the increase in the viscosity. The presence of both tyrosinase and α-chymotrypsin in seawater is associated with maximum pH-dependent viscosity changes, at around pH 6.5. The effect of salinity levels in this system at pH 6.5 is depicted in Fig. 4. No change in the viscosity was observed in 4% simulated seawater; increasing the salinity accelerated the viscosity change to a maximum level at 20% simulated seawater. At above 33% seawater concentration the hydrophobic stacking effect between Tyr residues predominates, causing a rapid visible precipitation, as described in the explanation of results in Fig. 1.

Absorption spectroscopy was selected to monitor the reaction in the investigation of the auto-crosslink mechanism. A low molecular weight *t*-butoxycarbonyl (Boc)-L-β-3,4-dihydroxyphenyl-α-alanine (Dopa) quinone-α-Boc-L-lysine model system was prepared to confirm the explanation given for the results of Figs. 2—4 (the synthesis will be described elsewhere). The absorption spectra of Boc-Dopa quinone, Boc-L-Dopa quinone-α-Boc-L-lysine, poly(Lys Tyr) and crosslinked poly(Lys Tyr) containing tyrosinase and α-chymotrypsin in aqueous media are depicted in Fig. 5. The absorption spectra of Boc-L-Dopa quinone (curve A) exhibited an absorption band at 340 nm and a shoulder at around 270 nm. To this solution was added α-Boc-L-lysine (no absorption in the 230—450 nm wavelength range). The development rate of the new absorption band at 360—365 nm due to the

crosslink reaction was gradual and slowed down after overnight (curve B). Poly(Lys Tyr) exhibits an absorption peak at 275 nm (curve C). The addition of tyrosinase and α-chymotrypsin to the poly(Lys Tyr) solution caused a rapid 275-nm spectroscopic change (decrease) in seawater and turbidity after about 4 min, while the same system in water exhibited a much slower change. The precipitated materials were collected by centrifugation and partially hydrolyzed by an 8 mol dm^{-3} HCl solution for 3 days at 50 °C, while becoming a clear solution. Curve D in Fig. 5 exhibits an absorption band at 360—365 nm which agreed with the absorption result of curve B of the crosslinked model compound.

From the above-mentioned results, the oxidase enzyme tyrosinase was found to catalyze a succesive reaction from phenol to catechol and, finally, to *o*-quinone in both fresh water and seawater. The oxidation reaction is much faster toward tyrosine than toward tyrosine copolymer. α-Chymotrypsin, thought to exist in the integument and to act as a kind of activator of prephenoloxidase, randomly digests the tyrosine copolymer at aromatic residues and accelerates oxidation by tyrosinase, followed by rapid precipitate formation due to intermolecular crosslinking. This may be a possible insolubilizing mechanism of adhesive proteins in water systems.

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