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Synthesis of adhesive protein from the vitellaria of the liver fluke *Fasciola hepatica*

H. Yamamoto and **K. Ohkawa**

Institute of High Polymer Research, Faculty of Textile Science and Technology, Shinshu University, Ueda, Japan

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Summary. The polynonapeptide $(Gly\text{-}Gly\text{-}Gly\text{-}Tyr\text{-}Gly\text{-}Gly\text{-}Tyr\text{-}Gly\text{-}Lys)_n$, which is a precursor sequence of adhesive protein from the vitellaria of the liver fluke *Fasciola hepatica* has been synthesized by the fragment coupling, followed by polycondensation, and by cleavage of the protecting groups by hydrogen bromide. The synthetic adhesive protein was estimated to have the molecular weight of 10,100 (12 repeating units as nonapeptide) and was found to have satisfactory amino acid compositions. The Tyr residues of the synthesized precursor polynonapeptides can be converted to the Dopa residues by tyrosinase, giving the synthetic adhesive protein of the liver fluke.

Keywords: Amino acids – Adhesive protein – Polynonapeptide – Liver fluke

Introduction

Since 1983 the primary structures of some adhesive proteins secreted from the marine invertebrate have been determined by Waite (1990, 1985) as "consensus peptide sequences". The adhesive proteins are simple proteins and have been identified as L-β-3,4-dihydroxyphenyl-α-alanine (Dopa) and Lys containing proteins. The adhesive proteins have been investigated with the intention of applying the adhesive properties to medical and dental purposes (Waite, 1986; Benedict and Picciano, 1989). Two different approaches, one is polymer chemical and the other is gene technological, are competitive strategies to prepare these adhesive proteins. In fact, our group first synthesized a polydecapeptide $(Ala\text{-}Lys\text{-}Pro\text{-}Ser\text{-}Tyr\text{-}Hyp\text{-}Hyp\text{-}Thr\text{-}Dopa\text{-}Lys)_n$ from mussel *Mytilus edulis* (Yamamoto, 1987) and later polyheptapeptide $(Ala\text{-}Gly\text{-}Dopa\text{-}Gly\text{-}Gly\text{-}X\text{-}Lys)_m$ from Chilean mussel *Aulacomya ater* (Yamamoto et al., 1991) by polycondensation, and in the same time two groups prepared a precursor form Ala-Lys-Pro-Ser-Tyr-Pro-Pro-Thr-Tyr-Lys by genetic engineering technology (Maugh,

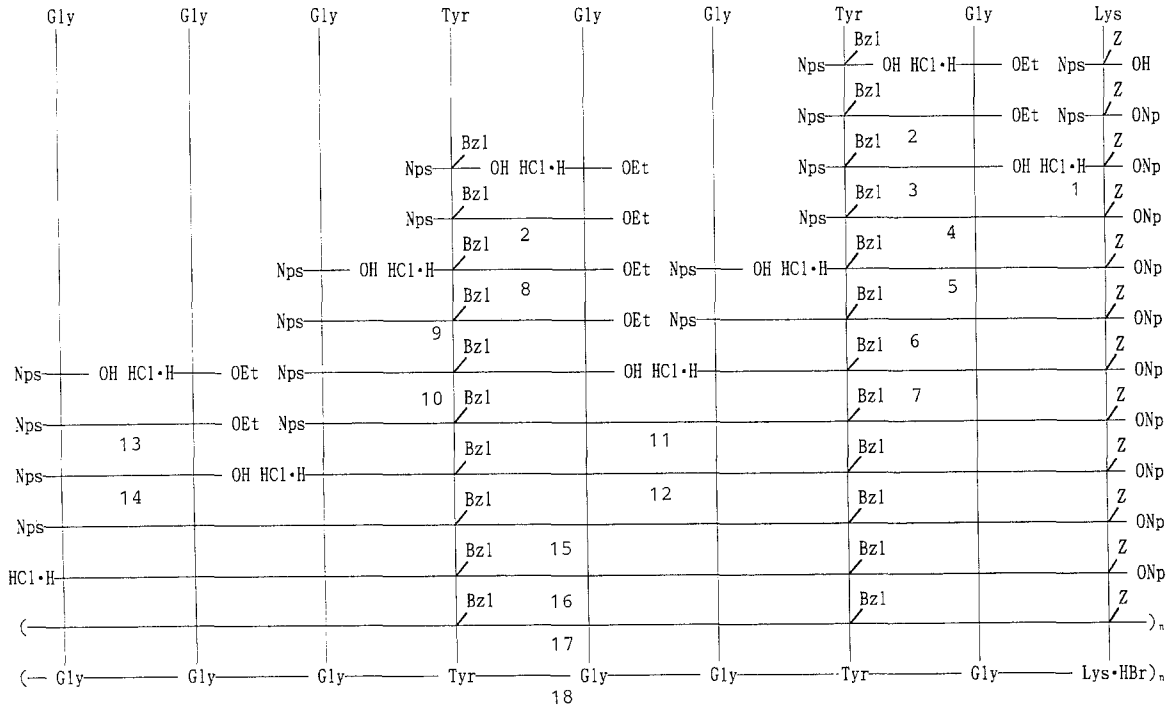
1984; Strasberg et al., 1989). The latter genetic products were converted to the final adhesive proteins by a modification reaction by tyrosinase.

Among the primary structures, the adhesive proteins from the egg shell hardening protein screlotin have also been reported. The adhesive protein of the vitellaria of the liver fluke *Fasciola hepatica* has the simplest repeating sequences consisting of only three amino acids (Gly, Tyr, Lys) among the adhesive proteins and therefore may be the most promising bioadhesive formulation (Waite and Rice-Ficht, 1987). The adhesive protein of the vitellaria of the liver fluke has been analyzed to have a sequence of (Gly-Gly-Gly-Dopa-Gly-Gly-Dopa-Gly-Lys) and a molecular weight of about 31,000 (about 35 nonapeptide units). In this article we report the synthesis of the precursor polynonapeptides (Gly-Gly-Gly-Tyr-Gly-Gly-Tyr-Gly-Lys) and the modification of the precursor polynonapeptides to final protein by the treatment of tyrosinase.

Experimental

Strategy

The synthesis of the liver fluke adhesive protein is outlined in Scheme 1. The combination of the ε-benzyloxycarbonyl (Z) and α-*o*-nitrophenylsulfenyl (Nps) groups was chosen to protect the amino groups of Lys, since the Z and Nps amino protecting groups can be selectively removable by a mild acidolysis, and ethyl (Et) and *p*-nitrophenyl (Np) carboxyl protecting groups were used. To protect hydroxyl groups of Tyr, the benzyl (Bzl) groups



Scheme 1. Preparation of poly(Gly-Gly-Gly-Tyr-Gly-Gly-Tyr-Gly-Lys). *Z* Benzyloxycarbonyl; *OEt* ethyl ester; *ONp p*-nitrophenyl ester; *Nps o*-nitrophenylsulfenyl; *Bzl* benzyl ether

were chosen. Hydrogen bromide was used for the simultaneous cleavage of O-Bzl and ε-N-Z protecting groups.

General procedure for synthesis

α-Nps groups of amino acid (Lys) or peptides were cleaved by the treatment of 2 equivalent molar 0.5–0.7 M hydrogen chloride in dry dioxane and the resulting hydrochlorides were recrystallized from alcohols and ether, or chloroform and ether (yield 67–94%). Et groups of peptides were saponified by the treatment of 1.4 equivalent molar 0.2–0.05 M sodium hydroxide in dioxane or tetrahydrofuran (THF), and the resulting acids were recrystallized from ethyl acetate and hexane (yield 70–95%). The fragment couplings were accomplished through the use of dicyclohexylcarbodiimide (DCCI) in dioxane, THF, chloroform and ethyl acetate, and the resulting peptides were recrystallized from ethyl acetate and hexane, or dioxane and hexane (yield 66–87%). All the reactions were monitored by TLC. TLC was carried out on silica gel plates (Merck Kieselgel G Type 60 GF_{254}) using the following solvent systems; A, chloroform-methanol-acetic acid (95 : 5 : 3, v/v); B, chloroform-methanol-water (90 : 10 : 1, v/v).

The viscosities were measured in dichloroacetic acid (DCA) and the molecular weight was estimated from the empirical viscosity equations.

Synthesis of adhesive protein in Scheme 1

Nps-Lys(Z), Nps-Tyr(Bzl) and Nps-Gly were prepared from the corresponding amino acids and Nps-Cl. After saponification of Nps-Tyr(Z)-Gly-OEt (2), HCl · Lys(Z)-ONp (1) was coupled with Nps-Tyr(Bzl)-Gly (3) to give Nps-Tyr(Bzl)-Gly-Lys(Z)-ONp (4, yield 87%) using DCCI. The compound 4 was converted to give HCl · Tyr (Z)-Gly-Lys(Z)-ONp (5, yield 92%) by treating with HCl/dioxane. The compound 5 was coupled with Nps-Gly to give Nps-Gly-Tyr(Z)-Gly-Lys(Z)-ONp (6, yield 93%). The compound 6 was converted to HCl · Gly-Tyr(Z)-Gly-Lys(Z)-ONp (7, yield 93%).

The compound 2 was converted to HCl · Tyr(Bzl)-Gly-OEt (8, yield 94%). The compound 8 was coupled with Nps-Gly to give Nps-Gly-Tyr(Bzl)-Gly-OEt (9, yield 66%) using DCCI. The compound 9 was saponified to give Nps-Gly-Tyr (Bzl) -Gly (10, yield 76%).

The compound 7 was coupled with compound 10 to give Nps-Gly-Tyr(Bzl)-Gly-Gly-Tyr(Bzl)-Gly-Lys(Z)-ONp (11, yield 69%) using DCCI. The compound 11 was converted to HCl · Gly-Tyr(Bzl)-Gly-Gly-Tyr(Bzl)-Gly-Lys(Z)-ONp (12), which was recrystallized from chloroform and ether to give the heptapeptide (12, yield 67%).

Nps-Gly-Gly-OEt (13, yield 84%), which was prepared from Nps-Gly and HCl · Gly-OEt, was saponified with 1 M NaOH to give Nps-Gly-Gly (14, yield 70%). The compound 14 was coupled with compound 12 to give Nps-Gly-Gly-Gly-Tyr(Bzl)-Gly-Gly-Tyr(Bzl)-Gly-Lys(Z)-ONp (15, yield 84%). The compound 15 was treated with HCl/dioxane to give HCl · Gly-Gly-Gly-Tyr(Bzl)-Gly-Gly-Tyr(Bzl)-Gly-Lys(Z)-ONp (16, yield 87%: CHN Found C, 59.8; H, 5.8; N, 11.9%). Calcd for $C_{64}H_{72}O_{16}N_{11}Cl$ C, 59.7; H, 5.6; N, 12.0%.

Polycondensation was accomplished in the following manner; the active ester hydrochloride 16 (1.3 g) was dissolved in 3.5 ml of dry dimethyformamide by the addition of 1.1 equivalent molar triethylamine and reacted for 10 days at room temperature. After precipitation by water and purification, poly[Gly-Gly-Gly-Tyr(Bzl)-Gly-Gly-Tyr(Bzl)-Gly-Lys(Z)] (17) was obtained in 88% yield: CHN Found C, 62.8; H, 6.1; N, 12.5%. Calcd for $C_{58}H_{66}O_{13}N_{10}C$, 62.7; H, 6.0; N, 12.6%; $[\eta] = 0.165$ in DCA at 25°C. The compound 17 (1.0 g) was treated with 30 fold hydrogen bromide/acetic acid coexisting 3 equivalent molar thioanisole for 60 h at 50°C to give final product poly(Gly-Gly-Gly-Tyr-Gly-Gly-Tyr-Gly-Lys) (18). The residual polynonapeptide was washed with dry ether and ether-ethanol, and dried. The resulting crude product was dissolved in methanol and precipitated by ether. The precipitate was dissolved in water, and the solution was dialyzed for 2 days against distilled water using cellulose dialysis tubing (molecular cut off 3,500) and lyophillized (yield, 90%); amino acid analysis $Gly_{6.19}Tyr_{1.94}Lys_{1.00}$.

Results and discussion

At the beginning we predicted the solubility of the synthetic intermediates during the synthesis might be the most difficult problem to be solved because of the high Gly content of the adhesive protein from the vitellaria of the liver fluke *Fasciola hepatica*. However, the adhesive protein precursor (17) was successfully synthesized by dividing the nonapeptide into three glycyl fragments, in which each contains every two Gly residues as shown in Scheme 1. The mean molecular weight of the adhesive protein precursor was estimated to be 12,800 (12 repeated nona-sequence). The amino acid composition of the deprotected protein (molecular weight 10,100; 18) agreed with the theoretical composition.

The synthesized nonapeptide 18 (2.2 mg) was dissolved in 3 ml of simulated seawater and treated with tyrosinase (42 units) at pH 8 and at 22°C. Figure 1 shows the spectroscopic change of the solution with time. The characteristic absorption band of Tyr at 278 nm shifted to 283 nm, to which Dopa residues have been assigned, after 20 min (shoulder) and 1 h (peak), thus giving the final adhesive protein of liver fluke. After 5 h, the Dopa residues in the nonapeptide sequence further changed to Dopa quinone judging from the new broad absorption band at around 350 nm. An insolubilization reaction succeeds and, after 80 h, the precipitates can be observed by the naked eye. The pH dependence profile

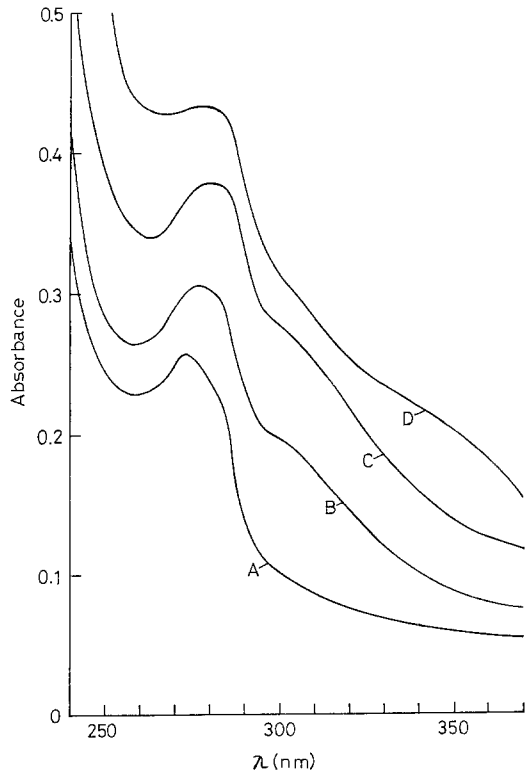


Fig. 1. Time-course of the absorption spectra of poly(Gly-Gly-Gly-Tyr-Gly-Gly-Tyr-Gly-Lys)$_{12}$ with tyrosinase at pH 8 and 22°C in simulated seawater: *A* 0 min; *B* after 20 min; *C* after 1 h; *D* after 5 h

of the apparent oxidation activity of tyrosinase toward the adhesive protein of liver fluke was examined. The optimal pH was found to be 8, and this agreed with the pH value of natural sea water (pH 8.2) and human haemolymph (pH 7.4).

Further studies of adhesive characteristics of the synthetic adhesive protein of liver fluke as bioadhesive formulations will be reported elsewhere.

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Authors' address: Dr. H. Yamamoto, Institute of High Polymer Research, Faculty of Textile Science and Technology, Shinshu University, Ueda 386, Japan.

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