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Pulcherosine, a Novel Tyrosine-Derived, Trivalent Cross-Linking Amino Acid from the Fertilization Envelope of Sea Urchin Embryo†

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ABSTRACT: The normally hardened and aminotriazole-induced soft fertilization envelopes (FEs) of the sea urchin *Hemicentrotus pulcherrimus* and two other species were isolated and investigated for component proteins and cross-linking amino acids. From the acid hydrolysate of the hard FE of *H. pulcherrimus*, we isolated by reversed-phase high-performance liquid chromatography a novel fluorescent compound as well as dityrosine and trityrosine, the major tyrosine-derived cross-linking amino acids. These three compounds were also isolated from the reaction products of the tyrosine/horseradish peroxidase/H_2O_2 system. The structure of the novel compound, designated "pulcherosine", was determined to be 5-[4″-(2-carboxy-2-aminoethyl)phenoxy]-3,3′-dityrosine. With respect to the position of diphenyl ether bond between the tyrosine and dityrosine moieties, it is an isomer of isotrityrosine found in *Ascaris* cuticle collagen [Fujimoto et al. (1981) *Biochem. Biophys. Res. Commun. 99*, 637–643]. Isotrityrosine was not found in either of the above systems as a major component. The contents of tyrosine, dityrosine, trityrosine, and pulcherosine in the hard FE of *H. pulcherrimus* were estimated as 255, 5.5, 2.1, and 1.3 residues, respectively, per 10 000 total amino acid residues, while in the soft FE, those of tyrosine and dityrosine were 305 and 0.25 residues, respectively, and trityrosine and pulcherosine were only traces. The molar ratio of dityrosine, trityrosine, and pulcherosine in the hard FE was 100:38:24, while that for tyrosine/horseradish peroxidase/H_2O_2 reaction products was 100:3:8, respectively.

One of the conspicuous processes in the early development of sea urchin is the elevation of fertilization envelope[1] (FE)[2] observed immediately after fertilization and its subsequent hardening. Within a minute postinsemination, the vitelline layer (VL), which is slightly modified from the state in the unfertilized eggs, elevates and associates, in a Ca^{2+}-dependent manner, with several proteins and enzyme(s) secreted from the cortical granules to make a soft precursor of FE (SFE). In 5–10 min it is converted to hard FE (HFE) by intermolecularly cross-linking the tyrosine residues in some of the assembled proteins and the VL-derived scaffold, forming dityrosine (DT), trityrosine (TT), and higher homologues by the action of ovoperoxidase, which is one of the components of SFE (Foerder & Shapiro, 1977; Hall, 1978; Somers & Shapiro,

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[1] Historically this investment has been called "fertilization membrane", although it is not a common biomembrane consisting of lipid bilayer and proteins, but rather a huge complex of glycoproteins and enzyme(s).

[2] Abbreviations: FE, fertilization envelope; HFE, hard fertilization envelope; SFE, soft fertilization envelope; VL, vitelline layer; DT, dityrosine; TT, trityrosine; PC, pulcherosine; IDT, isodityrosine; ITT, isotrityrosine; HRPO, horseradish peroxidase; HPLC, high-performance liquid chromatography; FAB, fast atom bombardment; MS, mass spectrometry; ATA, 3-amino-1,2,4-triazole; BA, benzamidine (hydrochloride); MFSW, Millipore-filtered seawater; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 2-ME, 2-mercaptoethanol; res, residue(s); TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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1989). The HFE, less permeable than SFE and resistant to physical forces, most proteolytic attacks, and disrupting agents, acts as a later block to polyspermy and protects the developing embryo. Half a day later, the embryo, in its early blastula stage, secretes a protease called hatching enzyme (EC 3.4.24.12; Ishida, 1936) to make an opening in HFE and swims out by the motion of cilia.

The cross-link dityrosine or 3,3′-bityrosine is an amino acid oxidatively derived from two free tyrosine molecules or tyrosine residues in proteins by the formation of a biphenyl-type structure. Since it was first found in resilin, a rubber-like protein of an insect wing ligament (Andersen, 1963, 1964, 1966), DT has been isolated from various structural proteins and hard tissues such as elastin (LaBella et al., 1967), collagen (LaBella et al., 1968), fibrin and keratin (Raven et al., 1971), cuticlin from *Ascaris* cuticle (Fujimoto, 1975), bivalve bysuss (DeVore et al., 1978), cataractous human lens protein (Garcia-Castinerias et al., 1978), and merino wool (Otterburn et al., 1984). A very high content of DT was reported in yeast ascospore wall (Briza et al., 1986). Its homologues, TT and tetratyrosine, were also found in resilin (Andersen, 1966), and brominated DT and TT were found in a crab exocuticle (Welinder et al., 1976). Another type of cross-link, 3,3′-methylenebis[tyrosine], was isolated from the hinge ligament protein of molluscs (Andersen, 1967). Isodityrosine (IDT) with a diphenyl ether bond between two tyrosine units was isolated from extensin, a plant hydroxyproline-rich protein (Fry, 1982), and isotrityrosine (ITT) with a phenyl ether bond between tyrosine and DT units was found in *Ascaris* cuticle collagen (Fujimoto et al., 1981).

Prior to the finding of DT in natural sources, DT and dityramine had been enzymatically synthesized from tyrosine and tyramine, respectively, by the horseradish peroxidase (HRPO)/H_2O_2 system (Gross & Sizer, 1959). Various structural and globular proteins and enzymes were modified by the above system, and DT was detected in the products (Aeschbach et al., 1976; Tressel & Kosman, 1980). Furthermore, UV irradiation, tetranitromethane, ozone, and periodate have been reported to cause DT formation (Lehrer & Fasman, 1967; Williams & Lowe, 1971; Verweij et al., 1982; Hsuan, 1987).

Studies on other aspects of the tyrosine-derived cross-links have also been performed: e.g., kinetics and stereospecificity of DT production by HRPO and lactoperoxidase (Bayse et al., 1972), separation and quantification by HPLC (Zaitsu et al., 1981; Otterbahn & Gargan, 1984), and NMR studies (Ushijima et al., 1984; Briza et al., 1986). However, most authors focused their interest and attention on DT only and did not extend their studies to other cross-links in relatively smaller amounts.

In the course of studies on the structure of the FEs of a few species of sea urchin, we found, by successful use of reversed-phase HPLC with a fluorescence monitor, a novel cross-linking amino acid with fluorescence typical of the compounds of DT family. It turned out to be identical to an unknown fluorescent compound in the products of the tyrosine/HRPO/H_2O_2 system. In this article, we describe isolation from both sources, characterization, and the structure of a new cross-linking amino acid which we designated pulcherosine (PC). We also did quantitative analyses of the three major tyrosine-derived cross-linking amino acids, DT, TT, and PC.

Materials and Methods

Materials. L-Tyrosine was a gift of Ajinomoto Co., Inc. (Tokyo). The sources of other chemicals were as follow: acetyl-L-tyrosine, glycyl-L-tyrosine, Trizma base, HRPO (lot no. 125F-9645), benzamidine hydrochloride (BA), and 3-amino-1,2,4-triazole (ATA) from Sigma Chemical Co. (St. Louis, MO); D-tyrosine, H_2O_2, ninhydrin spray, acetonitrile (HPLC grade), and other reagents of analytical grade from Wako Pure Chemical Industries, Ltd. (Osaka); trifluoroacetic acid (TFA) for HPLC from Nacarai Tesque (Kyoto); cellulose phosphate P11 from Whatman (Maidstone, England); deuterium oxide from Merck (Darmstadt, FRG); ninhydrin and amino acid standard mixture from Pierce Chemical Co. (Rockford, IL); and SDS–PAGE molecular weight standards H and L from Bio-Rad Laboratories (Richmond, CA).

Preparation of Fertilization Envelopes. The sea urchins *H. pulcherrimus* and *Anthocidaris crassispina* were collected in the vicinity of Noto Marine Laboratory on the Japan Sea coast, and *Glyptocidaris crenularis* urchins were collected from Aomori Bay by courtesy of Asamushi Marine Biological Station. The gametes were shed by injecting 0.5 M KCl into the body cavity. The egg suspension in paper-filtered seawater was passed through gauze and titrated to pH 5.5 with 0.1 N HCl for 2–3 min to remove the jelly layer and then readjusted to pH 8.2 witth 0.1 NaOH. The eggs were washed twice with paper-filtered seawater by decantation, aspiration, and resuspension and were finally suspended in Millipore-filtered (0.45-μm membrane) seawater (MFSW). The eggs were then fertilized with 100× diluted "dry" sperm and incubated with gentle stirring for 30 min at room temperature (about 10 °C in March). The embryos were allowed to settle, and the supernatant was removed by decantation and aspiration, washed with MFSW twice, and then centrifuged at low speed (6*g*). The pelleted embryos were suspended and homogenized with a Teflon pestle homogenizer for about 10 strokes in distilled water containing 10 mM BA, which was added to suppress the possibly remaining activity of trypsin-like protease(s) from embryo cytosol and cortical granules. The homogenate was centrifuged at 600*g* for 5 min. The pellet was resuspended and homogenized in 10 mM BA. This homogenization/centrifugation procedure was repeated 10–15 times until the orange color of the supernatant was faint and the pelleted FEs were white. Alternatively, MFSW containing 10 mM BA (MFSWB) was used instead of simple 10 mM BA as homogenization medium. In this case distilled water was used once or twice to dissolve contaminating hyaline layers. Upon addition of MFSWB and homogenization, large jelly aggregates formed that were easily picked out. These aggregates could be used as starting material for isolation of hyalin, a major Ca^{2+}-insoluble glycoprotein component of hyaline layer (Stephens & Kane, 1970). When the removal of the orange color was difficult, MFSWB containing 0.01% Triton X-100 was used once or twice during the procedure. The finally pelleted FE was suspended in 50 mM Tris-HCl (pH 7.5) containing 10 mM $CaCl_2$ and 10 mM BA (buffer A) and further purified by discontinuous sucrose density gradient centrifugation with layers of 30, 40, 50, and 60% sucrose in buffer A at 3000*g* for 30 min. Most FEs concentrated at the interface of the 50 and 60% layers. They were collected with a pipet, washed with 1 mM $CaCl_2$ and then quickly with distilled water, and finally lyophilized. At each step of the procedure purity of FE was checked by light microscopy with attention to small particles from the embryos and hyaline layer aggregates.

Soft FE essentially devoid of the tyrosine-derived cross-links was prepared by fertilizing the eggs in the presence of 2 mM ATA, an inhibitor of peroxidases. At 30 min postinsemination, the embryos were wased wiith MFSW containing 2 mM ATA and 10 mM BA (MFSWAB) twice and passed two or three

times to strip the SFE off through two- or four-layered nylon cloth with 100-μm mesh opening (NRK, 13-XX), which was set at the widely cut needle end of a 50-mL disposable syringe. The embryo suspension was centrifuged at low speed (6*g*), and the SFEs that remained in the supernatant or softly layered over the denuded embryos were collected with a pipet. The embryos were resuspended in MFSWAB, and the procedure was repeated several times until no more SFE was layered over the embryos. The collected SFE was purified by discontinuous sucrose density gradient centrifugation in the same way as HFE, washed with 1 mM $CaCl_2$, washed quickly with distilled water, and lyophilized.

Another soft FE, designated "Ac-Tyr-FE", was prepared in a similar way to ATA-induced SFE using 5 mM Ac-L-tyrosine instead of 2 mM ATA during fertilization and the early stages of the isolation procedure.

Polyacrylamide Gel Electrophoresis. PAGE in the presence of 0.1% SDS was performed by using 3% stacking and 11% separating gels by the method of Laemmli (1970). The various FEs suspended in sample buffer containing 2% 2-ME were heated at 100 °C for 10 min, and insoluble materials were removed by centrifugation in an Eppendorf microfuge. Samples of 40 μg were loaded, and protein bands were stained with Coomassie Brilliant Blue R-250.

Isolation of Tyrosine-Derived Cross-Links. The lyophilized HFEs prepared from *H. pulcherrimus* were hydrolyzed with constant-boiling HCl (5.7 N) containing 0.02% phenol in a test tube placed in a small desiccator at 110 °C for 20 h under reduced pressure. The hydrolysate was dried over NaOH pellets in a vacuum desiccator, redissolved in 0.02 N HCl, and Millipore-filtered (0.45-μm membrane) by centrifugation. The filtrate was loaded on a cellulose phosphate column (Whatman P11, 0.9 × 12 cm) equilibrated with 0.2 M AcOH and eluted with a linear gradient of NaCl (0–0.5 M in 0.2 M AcOH, 150 mL + 150 mL) at a flow rate of 15 mL/h. The effluent was monitored by absorbance at 280 nm, and fractions of 3 mL were collected. An aliquot of each peak fraction as well as the starting hydrolysate were checked for fluorescent compounds by HPLC on a reversed-phase column (ODS, Erma-Hypersil 6 × 250 mm, or C8, Senshupak 8 × 250 mm). Elution was carried out with a linear gradient of acetonitrile (0–30%, 30 min) in 0.1% TFA. The apparatus, a Shimadzu LC-4A chromatograph with UV detector SPD-2AS (225 nm) and fluorescence detector RF-530 (Ex 283 nm, Em 410 nm) connected in tandem, and the integrators Shimadzu C-R1A and Hitachi D-2000 were used. The major two fluorescent peaks were identified as DT and TT on the basis of the data of MS and UV/fluorescence spectra, with reference to the previously reported ones (Andersen, 1963, 1964) as shown under Results. The third largest fluorescent peak (X) was not identified as any of the reported compounds. It was further purified by HPLC as described above to determine its structure. The peaks of the compound X from the repeated runs of HPLC were collected, combined, and lyophilized. The purified compound X was then subjected to the analyses described in the following sections.

Enzymatic Preparation of Tyrosine-Derived Cross-Links. Tyrosine-derived cross-links were synthesized from L-tyrosine by the action of HRPO/H_2O_2 basically by the method of Gross and Sizer (1959) with slight modification. Tyrosine (181 mg, 1 mmol) was dissolved in 100 mL of 50 mM sodium borate (pH 9.0), to which 3 mg of HRPO and 1 mL of 3.5% H_2O_2 (1.03 mmol) were added, and incubated at 37 °C for 20 h. At 1-h intervals during incubation, aliquots of 20 μL were withdrawn and subjected to HPLC as described above to check the progress of reaction and the nature of products. After incubation the reaction mixture was lyophilized and redissolved in 35 mL of distilled water. Insoluble materials, mainly sodium borate and unreacted tyrosine, were removed by centrifugation, and the dark brown supernatant was titrated to pH 2 with 6 N HCl. The supernatant was subjected to chromatography on a cellulose phosphate column (1.6 × 12 cm), and elution was carried out similarly to that of the hydrolysate of sea urchin FE. Major fluorescent peaks were collected, lyophilized, and further purified by HPLC as described above.

Mass Spectrometry. Mass spectra were obtained by using a JEOL DX304/DX304 tandem mass spectrometer at 3-kV accelerating voltage. Xenon (99.998%) was used as the source of the fast atom beam (3 keV), and glycerol was used as the liquid matrix for FAB ionization of the cross-links.

Nuclear Magnetic Resonance Spectra. NMR spectra were recorded on a Varian VXR-400S spectrometer, operating at a magnetic field of 9.4 T, under a ^1H frequency of 399.952 MHz and a ^{13}C frequency of 100.577 MHz. Sample concentrations of 8.3 mM were used in D_2O, and the spectra were taken by using 5-mm tubes. The solvent was used for a field frequency lock, and the methylene resonance of dioxane, taken as 3.75 ppm for ^1H and 69.19 ppm for ^{13}C from sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), was used as a secondary internal chemical shift reference. Proton NMR spectra were acquired in the quadrature phase detection mode with 30K data points, 20° pulse width, and a spectral width of 6000 Hz. The strong solvent signal (H_2O at 4.85 ppm) was presaturated during a 2.5-s relaxation delay. Data for the signals are reported as follows: chemical shift (ppm), integration, multiplicity (d = doublet, dd = doublet of doublet, m = multiple), coupling constant (Hz), and assignment. ^{13}C NMR spectra were taken with broad-band proton decoupling, employing the 35° pulse width, 0.2-s delay, and 30K data points. DEPT (distortionless enhancement by polarization transfer) spectra were determined to assign carbon multiplicities. Data for signals are given in the pair of chemical shift (ppm) and multiplicity (s = C, d = CH, and t = CH_2). The ^1H–^{13}C HETCOR (heteronuclear chemical shift correlation) experiment was carried out with J_{C-H} = 140 Hz.

UV Absorption and Fluorescence Spectra. UV spectra were recorded on a Shimadzu UV 160 or a Cary 219 spectrophotometer for 200–400-nm wavelength. Stock solutions of each cross-linking amino acid were diluted to 0.2 mM with 0.02 N HCl or 0.02 M NaOH. The molar extinction coefficient of compound X (pulcherosine) was calculated on the solution with known concentration determined by the method described under Quantitative Analyses of Tyrosine-Derived Cross-Links. The excitation and emission fluorescence spectra of the same solutions were recorded on a Shimadzu RF-5000 spectrofluorometer under a spectral bandwidth of 5 nm.

Alkaline Hydrolysis. Ten microliters of each solution of cross-links was taken into a polypropylene tube (12 × 75 mm) containing 100 μL of 4.4 N KOH, placed in a siliconized small desiccator, and hydrolyzed at 110 °C for 20 h under reduced pressure. The hydrolysate was neutralized with 6 N HCl, diluted with 0.02 N HCl, and subjected to amino acid analysis and HPLC.

Amino Acid Analysis. Samples prepared by acid or alkaline hydrolysis were dissolved in or diluted with 0.02 N HCl and automatically injected onto the column (4 × 150 mm) of a Hitachi 835-50 automatic amino acid analyzer. Analyses were performed by using citrate buffers with the program for high resolution of amino acids in protein hydrolysates. Total amino acid content of each FE sample was shown in nanomoles, and

particularly, tyrosine content was shown in res/10 000 total amino acid res.

Thin-Layer Chromatography. A cellulose thin-layer sheet, Chromagram 6064 (Eastman), solvent A (1-butanol/acetic acid/water, 4:1:2 v/v), and sovlent B (1-butanol/pyridine/acetic acid/water, 15:10:3:12 v/v) were used. Amino acids were located by spraying the thin-layer sheet with 0.5% ninhydrin in 1-butanol followed by drying and heating with a hair dryer.

Quantitative Analyses of Tyrosine-Derived Cross-Links. Quantification of DT, TT, and PC was performed by HPLC under UV and fluorescence monitoring in parallel with amino acid analysis. The content of each cross-link was shown in res/10 000 total amino acid res.

The absolute concentration of each standard solution of DT, TT, and PC was determined by the trinitrobenzenesulfonic acid (TNBS) method (Satake et al., 1960) for free amino group quantification, with calibration on tyrosine solution (0–80 nmol). A standard mixture solution of DT, TT, and PC (1 μM each) was made, and 10 μL (10 pmol) was injected on the HPLC column for calibration.

As an alternative method, DT and TT for the standard solutions were made from the compounds *N,N'*-diglycyl-DT and *N,N',N''*-triglycyl-TT, respectively, which were prepared from glcyl-L-tyrosine by the action of the HRPO/H_2O_2 system. Hydrolysis of these compounds with HCl yielded mixtures of glycine/DT and glycine/TT, respectively, and the absolute concentrations of DT and TT in the mixtures were determined by amino acid analysis for glycine contents, assuming a yield of exactly 2 mol of glycine for 1 mol of DT and 3 mol of glycine for 1 mol of TT, respectively. We could not prepare the compound *N,N',N''*-triglycylpulcherosine from the reaction mixture due to its very low content and difficulty of isolation.

Results

Isolation of Fertilization Envelopes. The normally hardened FEs of *H. pulcherrimus* were prepared by two methods with different homogenization media, and their purities were compared with respect to microscopical appearance and SDS–PAGE pattern. The FE prepared in 10 mM BA appeared, before lyophilization, as a clear and transparent "ghost" with a nick, almost free of small particles and hyaline layer aggregates. At the stage of the last centrifugation/homogenization, those prepared in MFSWB were slightly contaminated with small particles and small-sized aggregates of hyaline layer. However, after density gradient centrifugation, these two preparations were almost of the same purity as observed by light microscopy, and SDS–PAGE revealed almost no difference in protein band pattern between the two.

Treatment with Triton X-100 apparently exerted no effect on the SDS–PAGE band pattern as compared with nontreatment. Since we did not measure the exact density of each protein band by densitometry, there remains a possibility that every non-cross-linked protein was evenly solubilized or certain proteins were selectively solubilized to the extent that the different density was indistinguishable with naked eyes. However, we think the solubilization was only minimal, if any, and did not seriously affect the conclusion of this study.

Both SFE and Ac-Tyr-FE were expected to be devoid of tyrosine cross-linking between polypeptide chains and had a much collapsed shape than HFE but were almost free of contaminating particles and hyaline layer aggregates.

Component Proteins of Hard and Soft Fertilization Envelopes. On SDS–PAGE as depicted in Figure 1 (lanes 1 and 2), the major stained bands in *H. pulcherrimus* FE were of molecular weight 95K, 70K, 48K, 32K, and 15K, for both hard and soft FEs. However, in the >100K region, the difference was clear; SFE was stained heavily at 280K and 180K, while normal HFE showed almost no bands in this region but some dark stain on the tops of stacking and separating gels. This suggests that the proteins of M_r >100K were cross-linked with one another or with the VL scaffold by the action of ovoperoxidase and the resultant protein complexes of high molecular weight were excluded from the separating gel and even the 3% stacking gel. There is, however, a possibility that the proteins of M_r <100K were also cross-linked with the complexes to some extent.

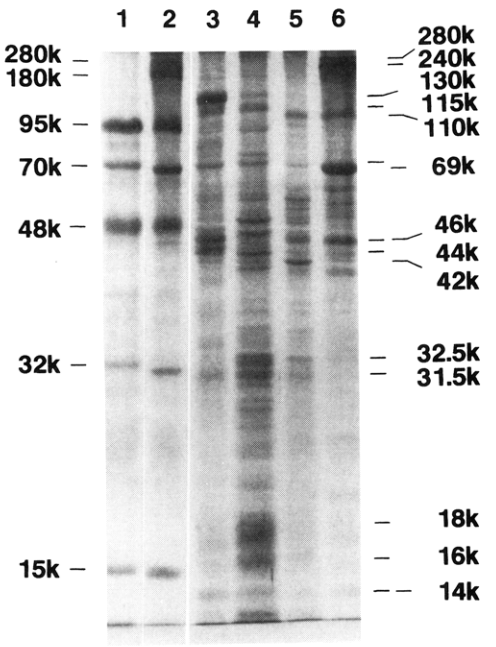

FIGURE 1: SDS–PAGE patterns of fertilization envelopes from three species of sea urchin. An 11% separation gel and a 3% stacking gel were used, and samples of 40 μg were loaded onto the gel. The protein bands were located by staining with Coomassie Brilliant Blue R-250. Lanes 1 and 2, hard and soft FEs, respectively, of *H. pulcherrimus*; lanes 3 and 4, hard and soft FEs, respectively, of *A. crassispina*; lanes 5 and 6, hard and soft FEs, respectively, of *G. crenularis*. Bio-Rad molecular weight standards H and L were used: myosin, 200 000; β-galactosidase, 116 250; phosphorylase B, 92 500; bovine serum albumin, 66 200; ovalbumin, 45 000; carbonic anhydrolase, 31 000; soybean trypsin inhibitor, 21 500; and lysozyme, 14 400.

On the basis of the results of our preliminary experiments on solubilization of these FE proteins, the 70K protein was identified as ovoperoxidase of this sea urchin species; namely, its cationic behavior on DEAE-Sephacel chromatography and its UV absorption spectrum typical of heme-containing protein (Soret absorption band at 414 nm) measured after EDTA/6 M urea/2-ME solubilization were very close to those reported by Deits et al. (1984).

The FEs of the other two species showed somewhat different band patterns. The HFE and SFE of *A. crassispina* (Figure 1, lanes 3 and 4) contained proteins of M_r 130K, 115K, 70K, 46K, and 44K. There appeared no clear difference in the high molecular weight region of both FEs. The FEs of *G. crenularis* (Figure 1, lanes 5 and 6) contained proteins of M_r 100K, 69K, 46K, and 42K as major ones. Its SFE had additional protein bands at 280K and 240K. The 69K and 70K bands of both species may also correspond to ovoperoxidase.

Cross-Links in Sea Urchin Fertilization Envelopes. Figure 2 depicts the HPLC profile of the HCl hydrolysate of *H. pulcherrimus* HFE. Major UV peaks without fluorescence were hydrophilic amino acids (5 min), tyrosine (18 min), and

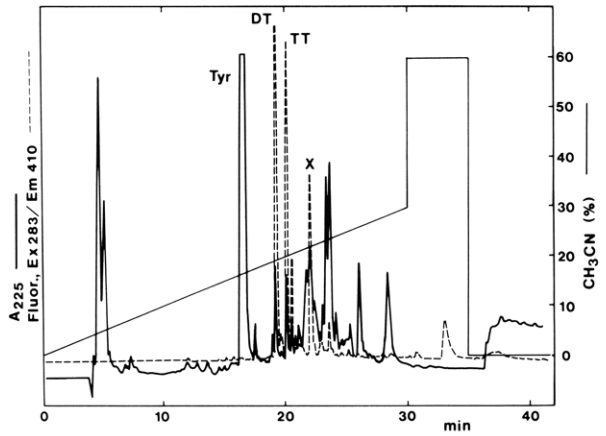

FIGURE 2: Separation of tyrosine-derived cross-links in *H. pulcherrimus* FE by reversed-phase HPLC. The acid hydrolysate of the FE (1.4 mg) was loaded on the column (ODS, 6 × 250 mm, Erma-Hypersil) and eluted by a linear gradient of acetonitrile (0–30% in 30 min) in 0.1% TFA at a flow rate of 2 mL/min. Effluent was monitored by absorbance at 225 nm (0.16 full scale, thick solid line) and fluorescence emission at 410 nm under excitation at 283 nm (range H128, broken line). Both detectors were connected in tandem.

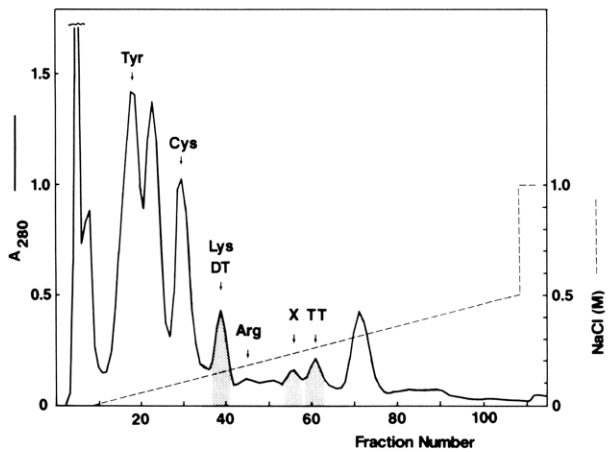

FIGURE 3: Separation of tyrosine-derived cross-links in *H. pulcherrimus* FE by cellulose phosphate column chromatography. The acid hydrolysate of the FE (75 mg) was loaded on the column (Whatman P11, 0.9 × 13 cm) equilibrated with 0.2 M AcOH and eluted with a linear gradient of NaCl (from 0 to 0.5 M, 300 mL) in 0.2 M AcOH at a flow rate of 15 mL/min. Effluent was monitored with absorbance at 280 nm, and fractions of 3 mL were collected. The elution position of some amino acids are shown by the arrows below their names. The shaded peaks contain the three major fluorescent compounds, which correspond to DT, X (PC), and TT shown in Figure 2. Brown pigments were eluted at the peak between the Tyr and Cys peaks.

some as yet unknown compounds (24–28 min). Among the three main fluorescent peaks, the two largest ones (19 and 21 min) were identified as DT and TT, respectively, by their UV spectral data in accordance with the previously reported ones (Andersen, 1966; Amado et al., 1984; Fujimoto et al., 1981) as shown in the following section and Table I, and by their FAB-MS data [$(M + H)^+$, 361 and 540, respectively]. The small peak eluted immediately after TT is presumably tetratyrosine. The third largest peak eluted at 22 min (compound X) was unknown, and for structural study, it was further purified by chromatography on cellulose phosphate and HPLC on a reversed-phase column. The profile of the cellulose phosphate chromatography is shown in Figure 3. The shaded three peaks with their top fractions 38, 56, and 61 were revealed by HPLC to contain fluorescent compounds that coincided with DT, X, and TT, respectively, as shown in the HPLC profile in Figure 2. The order of elution of X and TT

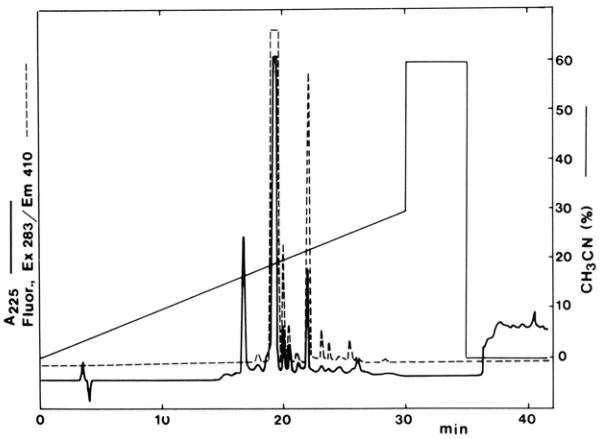

FIGURE 4: Separation of tyrosine-derived cross-links in the reaction products of the tyrosine/HRPO/H_2O_2 system by reversed-phase HPLC. The conditions of chromatography were the same as in Figure 2. The amount loaded on the column corresponds to about 0.3 mg of the starting material (tyrosine). The four major peaks are tyrosine, DT, TT, and X (PC).

from cellulose phosphate was reversed as compared with the HPLC profile. Each fraction (53–58) of the X-containing peak was further subjected to HPLC to isolate the compound X, effectively removing some contaminating substances. The peaks of compound X were collected, combined, and lyophilized and were used for the analyses in the next step.

Isolation of Enzymatically Synthesized Cross-Links. The reaction products of the tyrosine/HRPO/H_2O_2 system were analyzed by HPLC as shown in Figure 4. The elution profile of the fluorescent compounds was quite similar to that of the HCl hydrolysate of sea urchin FE (Figure 2); the three major fluorescent peaks were eluted at the same retention time. By UV and fluorescence spectra (acidic and alkaline pH's) and mass spectra, the compounds in the two largest peaks at 19 and 20 min were identified as DT and TT, respectively. The peak at 22 min gave the same spectra as the compound X in the sea urchin FE. The areas of the fluorescent peaks DT, TT, and X comprised 82.9, 3.6, and 10.6%, respectively, of the total fluorescent area. It is noteworthy that the content of TT is lower than X, in contrast to the sea urchin FE, wherein TT is more abundant than X. Isolation of each fluorescent compound in sufficient amount for various analyses was carried out by cellulose phosphate chromatography. The chromatogram (not shown) resembled very well that of sea urchin FE except for the size of peaks, relative amount of DT, TT, and X, and the absence of other proteinaceous amino acids. It should be noted that most of the byproducts, mostly with no or little fluorescence, were eluted in three large peaks before DT, that tyrosine was eluted in the second peak of the three, and that most of the compounds in these three peaks did not appear in the HPLC profile with monitoring at 225 nm. The fractions of the three major cross-link peaks were further purified by repeated runs of HPLC and lyophilized. Starting from 181 mg of tyrosine, about 20 mg of DT, 1.1 mg of TT, and 2.7 mg of X were obtained.

Ultraviolet and Fluorescence Spectra. The UV spectra of the compound X from sea urchin FE at both acidic (A) and alkaline (B) pH's are shown in Figure 5. The absorption maxima are 281.2 nm (pH 2.0) and 315.4 nm with a small shoulder at 255 nm (pH 12.0). The enzymatically synthesized X showed spectra almost identical with those of the natural compound, with maxima at 281.2 nm (pH 2.0) and 316.0 nm (pH 12.0). The molar extinction coefficient of X, measured on a Cary 219 spectrophotometer, was 8100 (281.2 nm, pH 2.0) and 10 500 (316 nm, pH 12.0). The absorption maxima

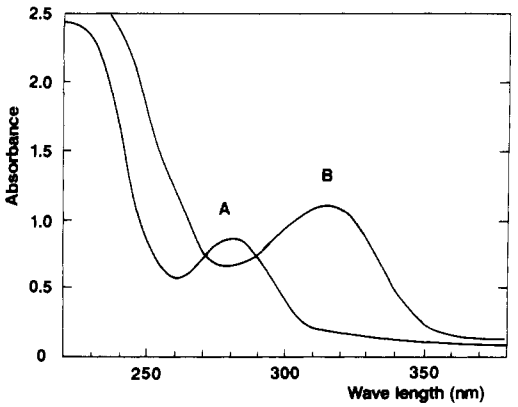

FIGURE 5: UV absorption spectra of pulcherosine. The solution of PC was diluted to 0.2 mM with 0.02 N HCl (pH 2.0) (A) or 0.02 M NaOH (pH 12.0) (B), and UV spectra were taken on a Shimadzu UV-160 spectrophotometer.

Table I: Spectroscopic Properties of Tyrosine-Derived Cross-Links

	UV absorption max (nm)		fluorescence Ex – Em maxima (nm)	
	pH 2.0	pH 12.0	pH 2.0	pH 12.0
PC (natural)	281.2	315.4	281–420	316–420
PC (synthetic)	281.2	316.0	281–420	318–422
DT (synthetic)	283	316	283–410	315–422
TT (synthetic)	286	321	288–412	323–409
DT[a]	283.5	316	283.5–415	316–415
DT[b]	284	317	222–405	315–415
TT[a]	286	322/315		
ITT[c]	280	300		
IDT[d]	273	298		
MBT[e]	278	298		
Br-DT[f]	286	315		
Br_2-DT[f]	288	316		
Br-TT[f]	288	314		

[a] pH 3.2/pH 11.1 for DT, and pH 2.5/9.8/13.0 for TT (Andersen, 1966). [b] 0.1 M HCl/0.1 M NaOH (Amado et al., 1984). [c] pH's not specified (Fujimoto et al., 1981). [d] pH 1/pH 13 (Fry, 1982). [e] 3,3′-Methylenebis[tyrosine]. pH's not specified (Andersen, 1967). [f] In 0.2 M AcOH and 0.1 M NaOH (Welinder et al., 1976).

of synthetic DT were 283 nm (pH 2.0) and 316 nm (pH 12.0), and those of TT were 286 nm (pH 2.0) and 321 nm (pH 12.0). Natural DT and TT gave the same results as the synthetic ones.

The fluorescence spectra of natural X (not shown) indicated its excitation/emission maxima to be 281.2 nm/420 nm at pH 2.0 and 316 nm/420 nm at pH 12.0. Synthetic X showed values very close to those of natural X: 281 nm/420 nm (pH 2.0) and 318 nm/422 nm (pH 12.0). Both X's had another excitation maximum at 240 nm with similar intensity to that of 281 nm. The values for the synthetic DT and TT were as follows: DT, 283 nm/410 nm (pH 2.0) and 315 nm/422 nm (pH 12.0); and TT, 288 nm/412 nm (pH 2.0) and 323 nm/409 nm (pH 12.0). These values coincide fairly well with the values previously reported by Andersen (1966) and Amado et al. (1984). The spectral data are summarized in Table I, with previously reported ones for comparison.

Amino Acid Analysis. By a high-resolution program DT was eluted as a broad peak between lysine and ammonia with poor separation from them, and TT and PC were eluted as extremely broad and flat peaks after ammonia and histidine due to strong interaction with the matrix of ion-exchange resin. Thus, quantification of DT by this method was barely possible for samples containing lysine, and that of TT and PC was impossible.

Alkaline Hydrolysis. Natural X as well as synthetic X, DT, TT, and crude reaction products were hydrolyzed with 4 N NaOH and were subjected to amino acid analysis and HPLC. Amino acid analysis of natural X yielded only tyrosine, along with a small peak of an unidentified compound eluted at the position of valine, and DT was not detected. On HPLC, the major component of the hydrolysate of natural X was tyrosine, as detected by UV, and the fluorescence monitor revealed a small peak of intact X and another peak of unidentified compound eluted at 20.2 min with less intense fluorescence. No trace of fluorescent peak of DT was detected. Synthetic X gave the same results. The hydrolysate of synthetic DT yielded on HPLC a peak with its top split into two very close ones at the position of intact DT. This is presumably attributed to mesomerization caused during alkaline hydrolysis. Synthetic TT, after hydrolysis, was eluted at the same position as intact TT without peak top splitting, unlike DT. Both DT and TT did not yield free tyrosine. These results indicate that compound X is distinct from ITT, which gave DT and dihydroxyphenylalanine on alkaline hydrolysis (Fujimoto et al., 1981).

Thin-Layer Chromatography. Development by solvent A yielded the following R_f values: Tyr 0.56, DT 0.39, TT 0.26, X 0.29. The values for Tyr, DT, and TT are slightly higher than those reported by Fujimoto et al. (1981), (Tyr 0.50, DT 0.25, TT 0.16, and ITT 0.18) presumably due to the different supporting media (paper vs thin layer). However, it is evident that both X and ITT have R_f values slightly higher than that of TT. By solvent B, the values were Tyr 0.57, DT 0.35, TT 0.19, and X 0.21.

Mass Spectrometry. On FAB-MS in the positive ion mode, synthetic DT showed a major signal of molecular ion $(M + H)^+$ of m/z 361, and it was degraded to various fragments, mainly of m/z 344, 315, 298, 287, 269, and 181 as detected by MS/MS method. These values correspond to the fragments produced by deletion of OH, COOH, $CHNH_2$, or CH_2, alone or in combination, and the tyrosine moiety. The largest peak was of the fragment with deletion of a single OH (m/z 344). Synthetic TT yielded an $(M + H)^+$ of m/z 540, and by the MS/MS method major fragments of m/z 522, 494, 483, 466, 448, 433, 421, 407, 388, 358, 295, 288, 286, 267, and 165 were obtained. Natural X also gave a molecular ion with m/z 540, and by the MS/MS method fragments of m/z 523, 494, 479, 467, 448, 433, 421, 361, 347, 305, 221, 197, 165, and 135 were obtained. Synthetic X gave the same molecular ion and major fragments as the natural compound. The most abundant fragment from TT and natural and synthetic X's was that of m/z 494, which was produced by deletion of a single COOH.

Based on the results of analyses described above, the identification of DT and TT as such was confirmed. Both natural and synthetic compound X's were concluded to be identical with each other and to have the same molecular mass as TT and ITT. The identity of both X's was also confirmed by their coelution on HPLC; the mixture of both natural and synthetic X's was eluted as a single peak (not shown). It was also confirmed by their identical NMR spectra shown in the next section. It can be concluded that X contains in its structure a DT moiety and a tyrosine moiety linked with a covalent bond labile to alkaline hydrolysis but resistant against acid hydrolysis at 110 °C, quite similar to ITT in *Ascaris* cuticle collgen. More information to determine its structure was obtained from the measurement of NMR spectra described in the following section.

^1H and ^{13}C NMR Spectroscopy. Figure 6a depicts the 400-MHz ^1H NMR spectrum of compound X. In the aliphatic region three sets of signals corresponding to $C_\alpha H$ and $C_\beta H_2$ were observed: 4.00–4.09 (3 H, m, Hj) and 3.06–3.28

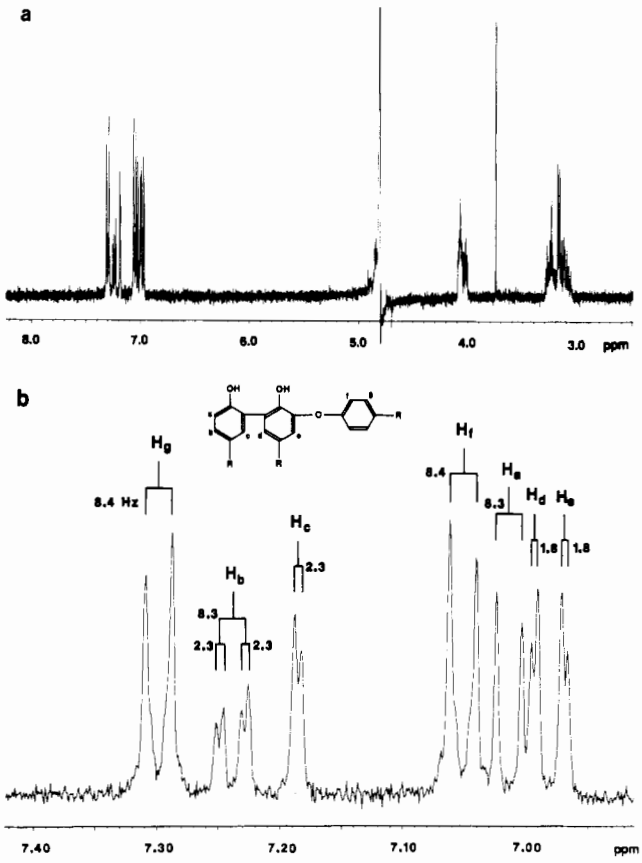

FIGURE 6: (a) 400-MHz ^{1}H NMR spectrum of pulcherosine. Dioxane was used as an internal standard (3.75 ppm). (b) Enlarged aromatic region of (a) with spin-coupling indications. The inset shows the numbering of the hydrogen atoms assigned in the predicted structure.

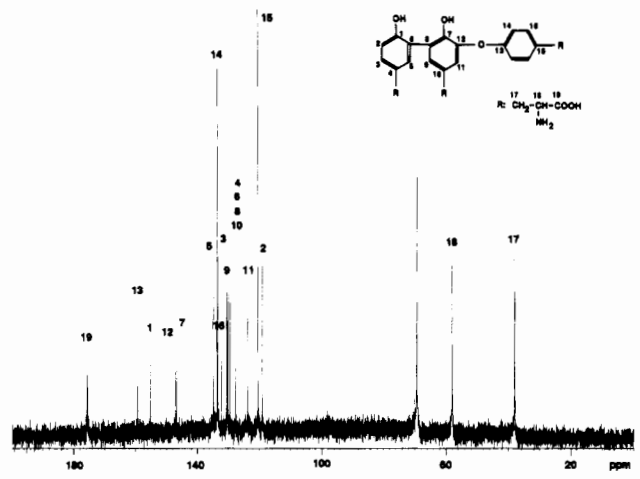

FIGURE 7: 100-MHz ^{13}C NMR spectrum of pulcherosine. As an internal standard, dioxane was added (69.19 ppm). The inset shows the numbering of the carbon atoms assigned in the predicted structure. Signals numbered as 17, 18, and 19 correspond to C_β, C_α, and C_{COOH}, respectively.

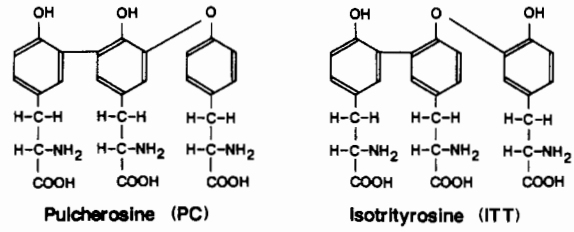

FIGURE 8: Structure of pulcherosine and isotrityrosine. The structure of ITT was reproduced from Fujimoto et al. (1981). Both compounds consist of DT and tyrosine moieties linked via phenyl ether bonds, but their positions or the origins of the phenolic oxygen forming the phenyl ether linkage are different.

ppm (6 H, m, Hi), respectively. In the aromatic region (Figure 6b, enlarged), nine protons were observed: 6.97 (1 H, d, J = 1.8 Hz, He), 6.99 (1, H, d, J = 1.8 Hz, Hd), 7.02 (1 H, d, J = 8.3 Hz, Ha), 7.06 (2 H, d, J = 8.4 Hz, Hf), 7.19 (1 H, d, J = 2.3 Hz, Hc), 7.24 (1 H, dd, J = 8.3 Hz, 2.3 Hz, Hb), and 7.30 ppm (2 H, d, J = 8.j Hz, Hg). The number of protons, nine, is one more than that of TT and is the same as ITT (Fujimoto et al., 1981). The peaks at 7.02, 7.19, and 7.24 ppm have the same chemical shifts and coupling constants as observed in the spectrum of DT by our own measurement (not shown) and as those previously reported by Ushijima et al. (1984) and Briza et al. (1986). This indicates the existence of a DT-type 1,2,4-trisubstituted benzene moiety in compound X.

In the ^{13}C NMR spectrum shown in Figure 7, a total of 23 signals appeared at the following chemical shifts: 37.80 (t), 37.87 (t), 37.95 (t), 57.89 (d), 58.05 (d × 2),118.99 (d), 120.43 (d × 2), 123.66 (d), 127.56 (s), 129.36 (s), 129.97 (s), 130.24 (s), 130.42 (d), 132.12 (s), 133.35 (d), 133.57 (d × 2), 134.82 (d), 146.73 (s), 147.16 (s), 155.16 (s), 159.28 (s), 175.46 (s), and 175.71 ppm (s × 2), corresponding to 27 carbons total.

The four signals of oxygen-substituted aromatic ring carbons (146.73–159.28 ppm) and nine aromatic protons suggest that the compound X is comprised of one DT moiety and one tyrosine moiety connected via a phenyl ether linkage in a similar way to ITT (Fujimoto et al., 1981). However, it is distinguished from ITT by the characteristic two doublets at 7.06 and 7.30 ppm (J_{ortho} = 8.4 Hz) in its ^{1}H NMR spectrum, and by ^{13}C signals at 120.43 and 133.57 ppm, which correspond to the para-substituted benzene partial structure of tyrosine. This indicates that the phenolic oxygen in the tyrosine unit, but not in the DT unit, contributes to the formation of a phenyl ether linkage. The tyrosine unit is most probably lined to one of the two positions ortho to the OH groups of the DT unit.

The only structure consistent with the results of various analyses described above (alkaline hydrolysis, ^{1}H and ^{13}C NMR spectroscopy, etc.) was deduced to be 5-[4″-(2-carboxy-2-aminoethyl)phenoxy]-3,3′-dityrosine. This structural formula is shown in Figure 8 with that of ITT for comparison. The well-resolved first-order spectrum due to the aromatic protons (Figure 6b) satisfies the structure.

This structure was also supported by the prediction of ^{13}C chemical shifts (C1–C16) by incremental calculation on its component groups, DT, phenylalanine [data from Briza et al. (1986)], and the phenoxy group [data from Brietmaier and Voelter (1978)], based on the well-known substituent effects on benzene aryl carbons. Namely, the ^{13}C chemical shifts of C1 through C12 in the DT moiety were calculated from the X-mimicking structure in which the C12 position (ortho to one of the two hydroxyl groups in DT unit) was substituted by a phenoxy group. In the same way, those of C13 through C16 in the tyrosine moiety were calculated from the structure where the C13 position (para position of phenylalanine) was substituted by a phenoxy group. The predicted values coincided well with the observed values (in parentheses) as follow: C1, 156.6 (155.16); C2, 120.0 (118.99); C3, 132.8 (132.12); C4, 129.3 (129.36);[3] C5, 134.7 (134.82); C6, 129.1 (127.56);[3] C7, 147.2 (146.73); C8, 130.5 (129.97);[3] C9, 129.4 (130.42);

[3] These four carbon signals were not assigned separately, and therefore, these values are interchangeable among the four.

Table II: Contents of Tyrosine and Tyrosine-Derived Cross-Links in the Fertilization Envelopes of Three Species of Sea Urchin

	amino acid res/10000 total amino acid res			
	Tyr	DT	TT	PC
H. pulcherrimus				
HFE	255	5.5	2.1	1.3
SFE	305	0.25	t[a]	t
Ac-Tyr-FE	323	6.3	0.74	0.48
A. crassispina				
HFE	271	8.4	3.1	1.9
SFE	318	0.46	t	t
G. crenularis				
HFE	357	4.1	1.6	1.0
SFE	401	0.2	t	t

[a] t, trace.

C10, 130.7 (130.24);[3] C11, 123.4 (123.66); C12, 149.2 (147.16); C13, 160.0 (159.28); C14, 122.7 (120.43); C15, 133.8 (133.57); and C16, 132.7 (132.12). If the meta position of DT unit is assumed for the phenyl ether linkage, the predicted chemical shifts of C7, C9, C11, and C12 considerably deviate (>10 ppm) from the observed values. The assignments of the protonated carbons were further confirmed by the HETCOR experiment.

We designated the compound X as "pulcherosine" (PC), since it was first found in the FE of the sea urchin *H. pulcherrimus*.

Contents of Tyrosine and Tyrosine-Derived Cross-Links. The contents of tyrosine and the three major tyrosine-derived cross-links of the various FE samples were determined by amino acid analysis and HPLC (Table II). The contents of DT, TT, and PC in HFE of *H. pulcherrimus* were 5.5, 2.1, and 1.3 res, respectively, per 10000 total amino acid res. Soft FE, which we expected to be completely devoid of any cross-link, contained about 5% of the DT in HFE, presumably due to incomplete inhibition of ovoperoxidase during fertilization and SFE isolation procedure. Tyrosine contents in SFE and HFE are 305 and 255 res, respectively, per 10000 total amino acid res with the difference being 50, whereas the Tyr residues converted into the three major cross-links, DT, TT, and PC are 13 residues in total. The rest of the Tyr residues, 37 res, cannot completely be accounted for by the formation of other higher order cross-links with fluorescence as shown in the HPLC profile (Figure 2). This discrepancy can be compromised only by the possible oxidative conversion to as yet unidentified derivatives mostly without fluorescence typical of the DT family (Figures 2 and 3) rather than by destruction during acid hydrolysis.

Ac-Tyr-FE, another soft FE, contained a slightly higher amount of the three cross-links than HFE and a slightly larger amount of tyrosine than ATA-induced SFE. The cross-links in Ac-Tyr-FE are very probably formed between the protein-bound Tyr residues and Ac-Tyr, thus preventing the FE polypeptide chains from being cross-linked, and leaving the FE soft. Excess amounts of Ac-Tyr presumably worked as a scavenger to protect the Tyr residues in the FE polypeptides from conversion to as yet unknown derivatives. The cross-links were also found in the seawater medium of fertilization. They were similar to the reaction products of Ac-Tyr/HRPO/H_2O_2 as analyzed by HPLC (not shown). The cross-link contents of the medium (containing 5 mM Ac-Tyr) were determined after HCl hydrolysis as Tyr, 99.71%, and the total of the three cross-links, 0.29%. Among the three, DT comprised 91.2%, TT 1.8%, and PC 6.9%, or in molar ratio, they were 100:2:8. The molar ratio of the three cross-links DT, TT, and PC in *H. pulcherrimus* HFE was 100:38:24, in contrast to 100:3:8 for the tyrosine/HRPO/H_2O_2 system. The FEs from other two species showed similar values of tyrosine and cross-link contents.

Discussion

The sea urchin FE elevates and hardens within 10 min after fertilization and later is dissolved by hatching enzyme and possibly some other proteases. It serves as a simple model system to investigte the production, assembly, posttranslational modification, and degradation of an extracellular matrix. We first intended to analyze the FE component proteins to localize the tyrosine-derived cross-links DT and TT as well as the isopeptide cross-link formed by transglutaminase (Battaglia & Shapiro, 1987), and to add further information on the molecular mechanism of sea urchin FE assembly, which was recently reviewed by Somers and Shapiro (1989).

For isolation of HFE we used 10 mM BA as homogenization medium to avoid its proteolytic degradation by proteases possibly remaining after postinsemination washings. However, addition of such protease inhibitors did not seem to be essential, and rather distilled water seemed to be the best and simplest homogenization medium as used by Carroll and Baginski (1978). It was used to assure and accelerate the complete cell lysis and dissolution of hyaline layers. It is a thin layer composed of several glycoproteins associated in a Ca^{2+}-dependent manner, including hyalin with M_r 330K as a major one (Stephens & Kane, 1970). It surrounds the immediate outer surface of the developing embryo and acts as an adhesive substrate keeping the blastomeres associated with one another (Dan, 1960).

The HFE of *H. pulcherrimus* is a huge complex of proteins with M_r 95K, 70K, 48K, 32K, and 15K, and the high M_r scaffold excluded from the gel of SDS–PAGE. The SFE prepared from the embryos fertilized in the presence of the peroxidase inhibitor ATA showed additional bands in the M_r >100K region. This electrophoretic pattern is very similar to the pattern of *Strongylocentrotus purpuratus* (Carroll & Baginski, 1978; Weidman & Kay, 1986; Vater & Jackson, 1989). The absence of the stained bands in the M_r >100K region in HFE strongly suggests that those proteins are almost entirely cross-linked with one another or to the scaffold by the action of ovoperoxidase and H_2O_2. If *H. pulcherrimus* also has proteoliaisin (230 kDa in *S. purpuratus*; Weidman et al., 1985), it must be cross-linked in the same way as other >100K proteins. These conclusions are, of course, based on the assumption that ATA acted only as an inhibitor of ovoperoxidase. Santiago and Carroll (1985) showed by immunological technique that the proteins from cortical granules (paracrystalline protein fractions; Bryan, 1970) are not cross-linked to the VL-derived scaffold. At least proteoliaisin, however, should be interpreted as an exception. The confirmation of their finding on other species has not been done.

In other sea urchins, the band pattern were quite different; the major bands in HFE and SFE of *A. crassispina* were of M_r 130K, 115K, 70K, 46K, and 44k, and those in HFE and SFE of *G. crenularis* were of M_r 100K, 69K, 46K, and 42k with additional bands of M_r 280K and 240K in SFE. From these results we can conclude that the FE-constituting proteins originating from cortical granules are highly variable except for M_r 70K ovoperoxidase, which seems to be conservative among species and genuses.

The Tyr-derived cross-links, and hence, ovoperoxidase which catalyzes their formation, play essential roles in rendering the sea urchin FE resistant to physical forces. This was confirmed by the fact that a soft FE prepared from the eggs fertilized in the presence of ATA was easily removed by forcing them through nylon mesh, while normally hardened FE could not

be denuded by this procedure.

The tyrosine-derived cross-links found in sea urchin FEs have so far been only DT and TT (Foerder & Shapiro, 1977; Hall, 1978), while brominated DT and TT, methylenebis-[tyrosine], IDT, and ITT were found in other sources. In the experiments reported here, we isolated for the first time a novel tyrosine-derived trivalent cross-link contained in smaller amounts than DT and TT, determined its structure, and named it "pulcherosine". As shown in our experiment, isolation of PC, as well as DT and TT, and their sensitive quantification highly depended on the application of reversed-phase HPLC with fluorescence monitoring. Although some former authors reported the isolation and/or quantification of DT and TT, they did not extend their work to other tyrosine-derived cross-links. This is most probably because of their relatively lower contents than DT and TT, and also because reversed-phase HPLC, which is highly effective for separation of compounds of closely related structure, was not so popular at that time. Both authors [Foerder and Shapiro (1977) and Hall (1978)] used only cellulose phosphate column chromatography for separation and quantification of DT and TT. They reported unknown fluorescent compounds, but they did not isolate them for further analysis. Andersen (1966), too, described an as yet unknown fluorescent compound in resilin, eluted just prior to TT as its shoulder from the cellulose phosphate column. He isolated it on DEAE-cellulose but did not perform further experiments on its structure.

In our HPLC analyses of the HCl hydrolysates of FEs and the reaction products of tyrosine/HRPO/H_2O_2 system, there were several more small peaks with or without fluorescence eluted before and after PC. If sufficient amounts of these compounds are prepared and various analyses are carried out, some unique structures of tyrosine derivatives will be unveiled. These tyrosine derivatives would be a clue to elucidate a more detailed mechanism of tyrosine oxidation by peroxidase/H_2O_2 systems leading to cross-link formation. The reaction mechanism for the formation of these cross-links has been thought to involve phenoxy radicals (Gross & Sizer, 1959), and formation of a tyrosine cation radical was suggested for the tyrosine/HRPO/H_2O_2 system based on its chemiluminescence (Ushijima et al., 1985). Recently, generation of superoxides during the fertilization of sea urchin eggs was reported (Takahashi et al., 1989).

In our experiment, ITT, an isomer of PC, was found neither in sea urchin FE nor in the reaction products of the Tyr/HRPO/H_2O_2 system at least as a major component. It may be contained in small amounts as one of the minor fluorescent peaks shown in the HPLC profiles (Figures 2 and 4). Since no authentic ITT was available, comparison of PC and ITT with respect to chromatographic behavior (HPLC, TLC, cellulose phosphate, etc.) and some physicochemical properties was not possible. According to Fujimoto et al. (1981), the content of ITT was 0.6 res/1000 total amino acid res, and almost no DT and TT were found in *Ascaris* cuticle collagen. However, an appreciable amount of DT (ca. 3 res/1000 res) was found in cuticlin, another component of *Ascaris* cuticle, distinct from collagen. This appears quite peculiar, since DT, TT, and PC are contained as the three major cross-links in both sea urchin FE and the products of the tyrosine/HRPO/H_2O_2 system. This suggests that the three tyrosine residues in *Ascaris* collagen polypeptide chains are in a somewhat unusual spatial arrangement in favor of ITT formation. It is clearly seen by building CPK molecular models of the two isomers that the rotation along the phenyl ether bond axis is more restricted in ITT than in PC. As another possibility, the enzyme responsible for the formation of this cross-link may have a specificity and mechanism of action different from those of HRPO and ovoperoxidase. Indirectly supporting this possibility, a difference was reported in the stereospecificity of HRPO and lactoperoxidase in the reactions of DT formation from D- and L-tyrosines; the former coupled the D-isomer more readily than the L-isomer, and in contrast, the latter oxidized the L-isomer more readily than D-isomer (Bayse et al., 1972).

As noted above, the three major cross-links in sea urchin FE are the same as those in the tyrosine/HRPO/H_2O_2 system, i.e., DT, TT, and PC. However, the molar ratio of DT/TT/PC is 100:38:24 in HFE and 100:3:8 in the product of the tyrosine/HRPO/H_2O_2 system, with predominant DT in the latter system. When 1 equiv of H_2O_2 is added to free tyrosine, a predominance of DT over the higher order cross-links is produced. This is readily expected, since the probability of the encounter of two free tyrosine molecules (radicals) is much higher than that of DT, one of the products, and tyrosine molecules (radicals). By contrast, in HFE, both ovoperoxidase and the substrate FE polypeptide chains containing tyrosine residues are integrated into FE as major components and immobilized. Ovoperoxidase, thus, can exert its catalytic action only on the tyrosine residues in its vicinity. With sufficient H_2O_2 the enzymes must effectively catalyze the cross-linking of DT and tyrosine residues to produce TT, PC, and other higher order cross-links. This is only possible where the third and fourth tyrosine residues are accessible to the immobilized enzyme and DT.

Since a rather large proportion of HFE proteins with very low, if any, cross-link content was solubilized with EDTA/6 M urea/2-ME in our preliminary experiments, most of the cross-links are expected to be localized to the central layer of FE (Chandler & Kazilek, 1986), or, in other words, the vitelline scaffolds plus proteoliaisin and presumably some portion of ovoperoxidase.

The DT contents in other sources are in the range of 0.1–2060 res/10000 total amino acid res: 0.1 for collagen (LaBella et al. 1968), 0.3 for chick aorta elastin (Keeley et al., 1969), 2.8 for keratin (Raven et al., 1971), 4.8–10.5 for merino wool (Otterburn & Gargan, 1984), 28 for tussah silk fibroin (Raven et al., 1971), 30 for *Ascaris* cuticle cuticlin (Fujimoto, 1975), 95 for resilin (Andersen, 1963), 255 for bivalve byssus (DeVore & Gruebel, 1978), and 2060 for yeast ascospore wall (Briza et al., 1986) (values recalculated from the original data). It is not strange that collagen and elastin have low DT contents, because their tyrosine contents, particularly in collagen, are inherently low and, moreover, they have their own characteristic cross-links such as pyridinoline and desmosine, both derived from three and four lysine residues, respectively, by initial catalysis of lysyl oxidase [for reviews, see Eyre (1987) and Rosenbloom (1987)]. The DT contents of sea urchin FEs [5.5–8.4 res for the three species in our experiment and 9.3 and 18 for *S. purpuratus* (Hall, 1978; Foerder & Shapiro, 1977)] are relatively low among the hard tissue proteins other than collagen, elastin, and keratin. However, the three major cross-links DT, TT, and PC, with some other so far unidentified ones, as a whole, appear to be sufficient to make the FE resistant to physical and chemical disruptions, although isopeptide cross-links may also contribute to some extent.

It is of great interest to compare the tyrosine and cross-link contents of sea urchin FEs and of yeast ascospore wall (Briza et al., 1986). Of the 305 res of tyrosine (per 10000 total amino acid res) in *H. pulcherrimus* SFE, 255 res are left intact in

HFE, and only 17 res (5.9%) are converted to the three cross-links with the remaining 33 res being unknown. In contrast, yeast ascospore wall precursor has an unusually high tyrosine content of 3935 res (by our recalculation), and 3449 res among them (87.5%) are converted to DT as the most abundant amino acid, comprising 20.6 mol % of the spore wall protein. The presumptive yeast peroxidase appears to be very effective in catalyzing the oxidation of tyrosine residues to DT but unable to produce the higher order cross-links DT and PC, in contrast to the *Ascaris* enzyme catalyzing ITT formation.

It will also be of great interest and value to investigate the presence of DT, TT, PC, and some other unknown cross-links and their contents in various other sources. Furthermore, the primary and three-dimensional structure, cDNA cloning, specificity, and mechanism of action of the *H. pulcherrimus* ovoperoxidase, and other peroxidases participating in the oxidative conversion of tyrosine residues to cross-links and other unknown derivatives, are all left to be investigated.

Furthermore, the finding of DT in cataractous human lens protein (Garcia-Castinerias et al., 1978) aroused our interest in the mechanism of formation of the cross-links and the brown pigments in eye lens caused by the aging process, as well as UV irradiation and diabetes, and possible methods to suppress and remove them.

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