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Identification of a Novel Amino Acid, *o*-Bromo-L-phenylalanine, in Egg-Associated Peptides That Activate Spermatozoa†

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ABSTRACT: Eight sperm-activating peptides containing a novel amino acid were isolated from the egg jelly of the sea urchin *Tripneustes gratilla*. Accurate mass measurement of the peptide in FAB mass spectrometry showed that the mass of the novel amino acid residue was 224.978. On the basis of the isotopic ion distribution and the degree of unsaturation, the mass value indicated that the elemental composition of the amino acid residue was $C_9H_8O_1N_1Br_1$, suggesting that the novel amino acid was bromophenylalanine. Proton NMR spectroscopy, amino acid analysis, and RP-HPLC with three synthetic isomers of bromophenylalanine demonstrated that *o*-bromophenylalanine was the novel amino acid. Derivatization of the amino acid with Marfey's reagent, (1-fluoro-2,4-dinitrophen-5-yl)-L-alanine amide (FDAA), further indicated that the amino acid was the L-isomer. In other sperm-activating peptides isolated from the egg jelly of the sea urchin, both *m*- and *p*-bromophenylalanines were discovered. The presence of *m*-bromophenylalanine has not been previously reported in natural products, while *p*-bromophenylalanine is found in theonellamide F, an antifungal bicyclic peptide from a marine sponge.

The egg-conditioned media (egg jelly) of sea urchins contain sperm-activating peptides (SAPs)[1] that have several effects on sea urchin spermatozoa. They cause stimulation of sperm respiration through intracellular alkalinization (Suzuki et al., 1981, 1984a; Hansbrough & Garbers, 1981; Repaske & Garbers, 1983), transient elevations of cAMP, cGMP, and Ca^{2+} concentrations in sperm cells (Garbers et al., 1982; Suzuki et al., 1984b; Lee & Garbers, 1986; Schackmann & Chock, 1986), and transient activation of the membrane form of guanylate cyclase (Ramarao & Garbers, 1985; Ward et al., 1986). Recently, it has been reported that SAP-I, Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly (Suzuki et al., 1981; Garbers et al., 1982), one of the sperm-activating peptides isolated from the egg jelly of sea urchins *Hemicentrotus pulcherrimus* and *Strongylocentrotus purpuratus*, promotes the acrosome reaction of *H. pulcherrimus* spermatozoa as a specific cofactor (Yamaguchi et al., 1988, 1989; Shimizu et al., 1990), SAP-IIA (Cys-Val-Thr-Gly-Ala-Pro-Gly-Cys-Val-Gly-Gly-Gly-Arg-Leu-NH_2) (Suzuki et al., 1984b), has been shown to act as a potent chemoattractant for spermatozoa of the sea urchin *Arbacia punctulata* (Ward et al., 1985).

The genetic code specifies only 20 amino acids as monomer building blocks in protein synthesis. Posttranslational modifications, such as phosphorylation, glycosylation, methylation and halogenation, of amino acids lead to additional diversity in the final protein products. In the past decade, many different SAPs have been purified and sequenced from the egg jelly of various species of sea urchins (Suzuki, 1990) but none of them has contained a modified amino acid except leucine amide in SAP-IIA. In the present study, we isolated eight SAPs that contain a novel modified amino acid from the egg jelly of the sea urchin *Tripneustes gratilla*. The structure of the novel amino acid was determined by FAB mass spectrometry, ^1H NMR spectroscopy, amino acid analysis, and RP-HPLC. We also found one more novel amino acid and another modified amino acid in some SAPs isolated from the egg jelly of the same species.

Experimental Procedures

Experimental Animals and Chemicals. The sea urchin *T. gratilla* was collected along the Okinawan coast of the East China Sea near Sesoko Marine Science Center, University of the Ryukyus. The sea urchin *H. pulcherrimus* was collected at the coast of Toyama Bay near Noto Marine Laboratory. Marfey's reagent, (1-fluoro-2,4-dinitrophen-5-yl)-L-alanine

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[1] Abbreviations: ACN, acetonitrile; *B/E*, mass spectrometric scan method where flux density (*B*)/deflecting voltage (*E*) = constant; Br-Phe, bromophenylalanine; cAMP, cyclic adenosine 3′,5′-monophosphate; CID, collision-induced dissociation; cGMP, cyclic guanosine 3′,5′-monophosphate; DDW, deionized and distilled water; FAB, fast atom bombardment; FDAA, (1-fluoro-2,4-dinitrophen-5-yl)-L-alanine amide; HPLC, high-performance liquid chlorotography; NMR, nuclear magnetic resonance; RP, reverse phase; SAP, sperm-activating peptide; TFA, trifluoroacetic acid.

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amide (FDAA), was obtained from Pierce Chemical Co. (Rockford, IL). D- and L-phenylalanines and *o*-bromobenzyl bromide were purchased from Nacalai Tesque, Inc. (Kyoto, Japan), and *m*- and *p*-bromobenzyl bromides were products of Aldrich Chemical Co., Inc. (Milwaukee, WI). Acetonitrile (ACN) of HPLC grade, sodium of chemical grade, trifluoroacetic acid (TFA), diethyl acetamidomalonate, and other reagents of analytical grade were products of Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Authentic Samples.[2] Three isomers (ortho, meta, and para) of bromo-DL-phenylalanine were synthesized in our laboratory by condensation of the corresponding bromobenzyl bromide and diethyl acetamidomalonate according to the methods reported previously (Albertson & Archer, 1945; Zeller et al., 1965). Two enantiomers of *o*-bromophenylalanine were prepared by bromination of D- or L-phenylalanine (Faulstich et al., 1973).

Preparation of Bromophenylalanine-Containing SAPs. Eggs were obtained by intracoelomic injection of 0.5 M KCl and collected in filtered sea water. The egg suspension was adjusted to pH 5.0 with 0.01 N HCl to solubilize the jelly coat and then centrifuged at 200*g* for 10 min. The supernatant fluid was mixed with 2 volumes of 99% ethanol and then centrifuged at 10000*g* for 30 min at 4 °C. The supernatant fluid was then concentrated at 50 °C in vacuo, delipidated by chloroform extraction, and lyophilized. The residue was dissolved in a minimum volume of deionized and distilled water (DDW), and the solution was filtered with a Millex-GV filter (0.22 μm, Millipore Co.) and then applied to a reverse-phase column of HPLC.

There is a possibility that the bromide ion in sea water (67.3 mg/kg) (Turekian, 1969) and peroxides may produce chemical bromination of the peptides during the purification. Therefore, we separated the peptide-containing fraction from the salt fraction as soon as possible and used precautions so as not to generate peroxides in any step of the purification and acid hydrolysis of the peptides.[4]

High-Performance Liquid Chromatography. HPLC was carried out with a Shimadzu Model LC-6A chromatography system with reverse-phase columns. The column effluent absorbance was monitored at 225 nm with use of a Shimadzu SPD-6V spectrophotometer. We used the following programs for isolation of SAPs.

Program I: A column (Shim-pack PREP C-8, 5 μm, 20 × 250 mm) equilibrated with 5% ACN in 0.1% TFA in DDW was eluted with the equilibration solvent for 15 min and then eluted with 60% ACN in 0.1% TFA for the next 15 min, at a flow rate of 9.9 mL/min.

Program II: A column (Unisil C-8, 5 μm, 4.6 × 250 mm) equilibrated with 10% ACN in 0.1% TFA was eluted for 10 min with the equilibration solvent, followed by a linear gradient of ACN from 10 to 50% in 0.1% TFA over a 40-min time period, at a flow rate of 1.0 mL/min.

Program III: A column (Unisil C-8, 5 μm, 4.6 × 250 mm) equilibrated with 5% ACN in 5 mM sodium phosphate (pH 5.7) was eluted for 20 min with the equilibration solvent, followed by a linear gradient of ACN from 5 to 30% in 5 mM sodium phosphate (pH 5.7) over a 40-min time period, at a flow rate of 1.0 mL/min.

Program IV: A column (Unisil C-18, 5 μm, 4.6 × 250 mm) equilibrated with 10% ACN in 0.1% TFA was eluted with a linear gradient of ACN from 10 to 30% in 0.1% TFA over a 40-min time period, at a flow rate of 1.0 mL/min.

Program V: A column (Unisil C-18, 5 μm, 4.6 × 250 mm) equilibrated with 5% ACN in 5 mM sodium phosphate (pH 5.7) was eluted with a linear gradient of ACN from 5 to 30% ACN in 5 mM sodium phosphate (pH 5.7) over a 60-min time period, at a flow rate of 1.0 mL/min.

Program VI: A column (Unisil Q C-8, 5 μm, 4.6 × 250 mm) equilibrated with 10% ACN in 10 mM ammonium acetate (pH 6.8) was eluted with a linear gradient of ACN from 10 to 30% in 10 mM ammonium acetate (pH 6.8) over a 40-min time period, at a flow rate of 1.0 mL/min.

For separation of the three isomers of Br-Phe, HPLC was carried out with a column (LiChrosorb RP-18, 7 μm, 4.0 × 250 mm) equilibrated with 10% ACN in 0.1% TFA. Isomers were eluted with a linear gradient of ACN from 10 to 25% in 0.1% TFA over a 30-min time period, at a flow rate of 1.0 mL/min. Effluent absorbance was monitored at 260 nm.

Determination of Respiration-Stimulating Activity. Respiration-stimulating activity of the samples obtained from each purification step was routinely determined with use of *H. pulcherrimus* spermatozoa as described by Suzuki et al. (1981) since *T. gratilla* belongs to the order Echinoida and the peptide from sea urchins in the order should be effective for *H. pulcherrimus* (Suzuki et al., 1988).

Amino Acid Analysis. Samples were hydrolyzed for 20 h in constantly boiling HCl (5.7 N) at 110 °C in vacuo. Hydrolysate dissolved in lithium citrate buffer (pH 2.2) was analyzed with a Hitachi L-8500 amino acid analyzer with use of a Hitachi No. 2622 SC column (4.6 × 150 mm). Analysis was performed with use of a sodium citrate buffer system with the standard program of the analyzer.

FAB Mass Spectrometry. FAB mass spectra were obtained with a JEOL JMX-HX100 double-focusing mass spectrometer equipped with a FAB ion source and a data acquisition system, JEOL JMA-DA5000, as described by Takao et al. (1984). Experiments to obtain FAB mass spectra were carried out with a xenon atom beam source at 5-keV accelerating potential. Mass assignment was made with use of a mixture of CsI and KI (2:1 w/w) as a mass reference. A sample (100–500 pmol) was dissolved in 50% acetic acid in DDW (1 μL), and the solution was loaded on a stainless steel plate and mixed with glycerol (0.5 μL) and 1 M HCl (0.5 μL) on the plate. Accurate FAB mass spectra were measured as described by Takao et al. (1990).

Nuclear Magnetic Resonance Spectroscopy. Proton NMR spectra were recorded on a JEOL JNM-GX400 FT-NMR spectrometer, operating at a magnetic field of 9.4 T, under a ^1H frequency of 399.7843 MHz. Samples were dissolved in D_2O (pD 7.4) at the concentration of 0.5 mM (natural samples) or 50 mM (synthetic samples), and the spectra were taken in a 5-mm (i.d.) tube. The solvent was used for a field

[2] Elemental analysis and FAB mass spectrometry of synthetic Br-Phe were consistent with the proposed structures. Anal. Calcd for $C_9H_{10}O_2N_1Br_1$: C, 44.29; H, 4.13; N, 5.74. Found for *o*-Br-DL-Phe: C, 44.15; H, 4.08; N, 5.76. Found for *m*-Br-DL-Phe: C, 44.06; H, 4.08; N, 5.89. Found for *p*-Br-DL-Phe: C, 44.20; H, 4.10; N, 5.96. Found for *o*-Br-L-Phe: C, 44.24; H, 4.11; N, 5.80. Found for *o*-Br-D-Phe: C, 44.15; H, 4.07; N, 5.94. FAB mass spectrum ($[M + H]^+$). Theoretical *m/z* value of Br-Phe: 244.0. Observed *m/z* value of *o*-Br-DL-Phe, *o*-Br-L-Phe, *o*-Br-D-Phe, and *m*-Br-DL-Phe: 243.9. Observed *m/z* value of *p*-Br-DL-Phe: 244.0.

[3] When peptide TG-1 was subjected to sequence analysis with a gas-phase protein sequencer (Applied Biosystems Model 470A), the second cycle resulted in no identifiable PTH-amino acid and the amino acid sequence of the peptide was partially determined to be Gly-(X)-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly.

[4] It should be mentioned that using identical extraction and purification procedures employed in the present study, we obtained only corresponding non-brominated peptides from the egg jelly of the other sea urchins *H. pulcherrimus*, *Strongylocentrotus nudus*, *S. purpuratus*, and *Pseudocentrotus depressus* (Suzuki et al., 1988; Yoshino et al., 1989).

Table I: Amino Acid Compositions[a] and Mass Values[b] of Br-Phe-Containing Sperm-Activating Peptides Obtained from the Egg Jelly of the Sea Urchin *T. gratilla*

amino acid	TG-1	TG-2	TG-3	TG-4	TG-5	TG-6	TG-7	TG-8	TG-9	TG-10
Asp	2.03 (2)	1.91 (2)	2.11 (2)			1.09 (1)			2.07 (2)	1.71 (2)
Ser				0.89 (1)	1.71 (2)		0.73 (1)			
Gly	5.05 (5)	5.15 (5)	4.73 (5)	6.23 (6)	5.24 (5)	6.17 (6)	6.27 (6)	7.04 (7)	5.28 (5)	5.47 (5)
Val	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
Ile				0.98 (1)						
Leu	0.97 (1)	0.89 (1)	1.05 (1)		1.01 (1)	1.01 (1)	0.99 (1)	1.02 (1)	1.03 (1)	1.03 (1)
Phe(*o*-Br)	1.02 (1)	1.12 (1)	1.05 (1)	1.03 (1)	1.05 (1)	1.08 (1)	0.96 (1)	0.96 (1)		
Phe(*m*-Br)									1.10 (1)	
Phe(*p*-Br)										0.94 (1)
total	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)
$[M + H]^+$	970.2 (970.3)	969.2 (969.3)	971.1 (971.3)	885.1 (885.3)	915.1 (915.3)	913.0 (913.3)	885.1 (885.3)	855.2 (855.3)	970.2 (970.3)	969.1 (969.3)

[a] The composition is shown as a normalized value with valine to have one residue, and the number in parentheses refers to the number of residues in the peptide found by sequencing. [b] The mass value in parentheses is shown as theoretical mass value of the peptide.

frequency lock, and the methylene resonance of ACN, taken as 2.07 ppm for ^1H 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 32 768 data points, 45° pulse width, and a spectral width of 5000 Hz.

Determination of Enantiomeric Structure. The enantiomeric structure of the amino acid was determined by the method of Marfey (1984). FDAA derivatives were loaded on a RP-HPLC column (Unisil Q C-18, 5 μm, 4.6 × 150 mm) and eluted by a linear gradient of ACN (10–40% in 60 min) in 0.05 M triethylamine phosphate (pH 3.0) at a flow rate of 1.5 mL/min. Effluent absorbance was monitored at 340 nm.

Results

Isolation of Bromophenylalanine-Containing SAPs. The ethanol extract of *T. gratilla* egg jelly obtained from 1000 female individuals was concentrated, delipidated, and lyophilized as described in Experimental Procedures. The residue containing respiration-stimulating activity was dissolved in DDW and subjected to HPLC with use of Program I. Materials containing an unknown amino acid, which was monitored by amino acid analysis, were eluted with 60% ACN in 0.1% TFA, and the fractions were saved, lyophilized, and then subjected to HPLC with Program II. Four fractions containing the unknown amino acid were obtained. These fractions were purified further by HPLC with use of Program III. Eight different fractions containing the unknown amino acid were obtained. These fractions were purified further by HPLC with use of Program IV and V. Four active peptides (TG-1, -4, -5, and -6) and the free unknown amino acid were purified in final amounts of 232.3, 54.6, 58.6, 40.9, and 659.1 nmol, respectively. However, the other fractions were not pure, judged from the amino acid compositions. Therefore, the fractions were subjected to rechromatography with use of Program IV with a LiChrosorb RP-18 column (7 μm, 4.0 × 250 mm) and then Program VI. Two different active peptides (TG-2 and -3) were purified in final amounts of 56.3 and 17.7 nmol, respectively. However, the rest of one fraction was not pure. Further fractionation by HPLC with use of Program IV with a Unisil Q C-8 column (5 μm, 4.6 × 250 mm) yielded two active peptides (TG-7 and -8) in final amounts of 90.1 and 24.6 nmol, respectively. Amino acid compositions of the eight peptides (TG-1–TG-8) are shown in Table I.

FAB Mass Spectrometry. In order to identify the unknown amino acid, we carried out FAB mass spectrometry (Figure 1A). Peptide TG-1 gave a prominent pseudomolecular ion signal at m/z = 970.2. The observed mass value and the isotopic ion distribution were nearly identical with the theoretical value (970.3) and the ion distribution of Br-Phe-containing SAP-I, of which the elemental composition was $C_{38}H_{57}O_{14}N_{11}Br_1$. Another fragment ion was observed at m/z = 892.2 in the spectrum. The isotopic ion distribution indicated the absence of a bromine atom, and the value corresponded to $[M + H]^+$ of SAP-I that contains Phe instead of Br-Phe. Nakamura et al. (1985) reported that halogenated phenylalanine was partially reduced to phenylalanine by the FAB ion source in a mass spectrometer. These results suggest that the unknown amino acid is Br-Phe. To confirm the presence of Br-Phe in peptide TG-1, it was subjected to accurate mass measurement. A signal at m/z = 970.326 was observed, which was identical with the theoretical value (970.327). Subtracting the sum of the mass of the amino acid residues (determined by amino acid analysis and automated Edman degradation)[3] from the observed mass value showed the mass of the unknown amino acid residue to be 224.978. On the basis of the isotopic ion distribution and the degree of unsaturation, the value (224.978) indicated that the ele-

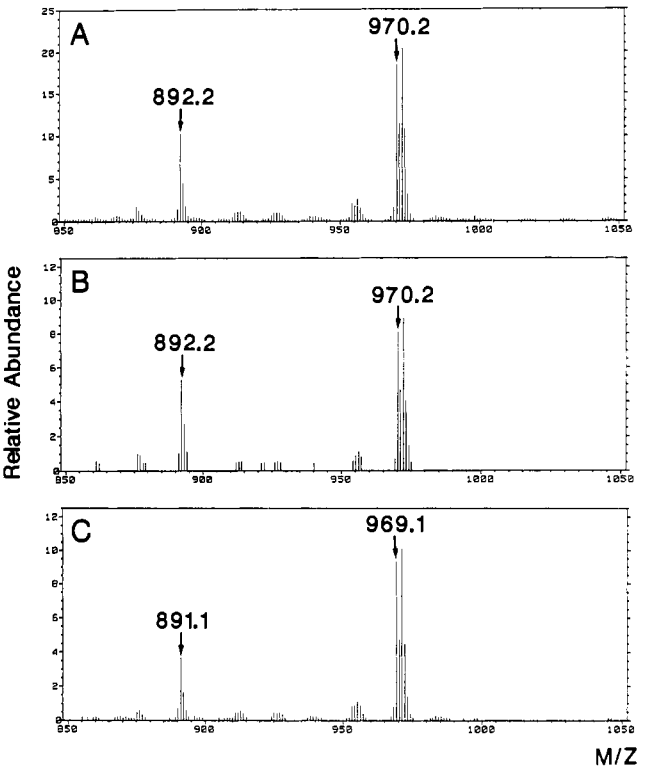

FIGURE 1: FAB mass spectra of SAPs TG-1 (A), TG-9 (B), and TG-10 (C), which contain an unknown amino acid.

Table II: Observed Chemical Shifts for the Benzene Protons of Three Synthetic Isomers of Br-Phe, Natural Free Br-Phe, and Br-Phe in Intact Peptide TG-1[a]

	synthetic *o*-Br-Phe	synthetic *m*-Br-Phe	synthetic *p*-Br-Phe	natural Br-Phe	intact peptide
H2		7.54 (s)	7.24 (d, J = 6.7)		
H3	7.71 (d, J = 7.9)		7.59 (d, J = 6.7)	7.70 (d, J = 7.6)	7.65 (d, J = 8.0)
H4	7.41 (t, J = 6.7)	7.56 (d, J = 8.2)		7.40 (t, J = 7.3)	7.35 (t, J = 7.6)
H5	7.29 (t, J = 7.3)	7.34 (t, J = 7.6)	7.59 (d, J = 6.7)	7.28 (t, J = 7.4)	7.23 (t, J = 7.2)
H6	7.39 (d, J = 6.7)	7.31 (d, J = 8.6)	7.24 (d, J = 6.7)	7.37 (d, J = 7.2)	7.29 (d, J = 8.0)

[a] Data for the signals are reported as follows. Chemical shifts are given in parts per million (ppm). For multiplicity, s = singlet, d = doublet, and t = triplet. Coupling constants (J) are given in hertz (Hz).

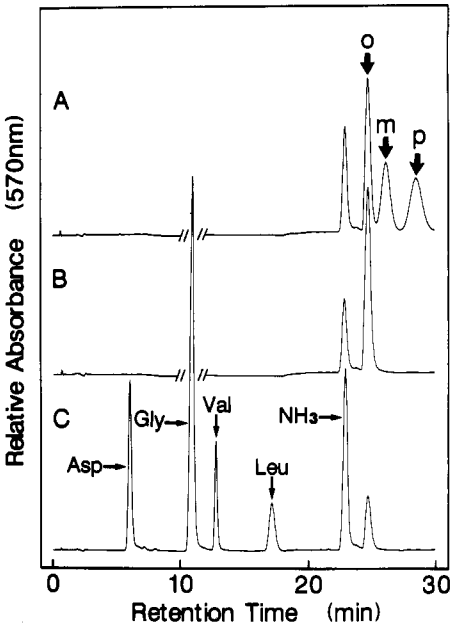

FIGURE 2: Detection of Br-Phe in egg jelly and SAP hydrolysates. (A) Automated amino acid analysis of the separation mixture of the three synthetic isomers of Br-Phe. An unknown peak eluted after NH_3 with a retention time corresonding to that of synthetic *o*-Br-Phe was found in (B) extracts of egg jelly and (C) the hydrolysate of peptide TG-1.

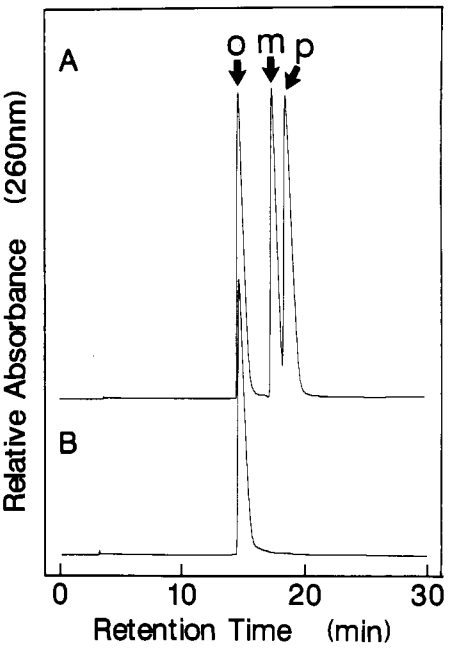

FIGURE 3: RP-HPLC profiles of the three synthetic isomers of Br-Phe (A) and free Br-Phe isolated from the egg jelly (B).

mental composition of the unknown amino acid residue was $C_9H_8O_1N_1Br_1$, consistent with the elemental composition of Br-Phe residue. FAB mass spectra of the peptides from TG-2 to TG-8 also indicated the presence of one Br-Phe residue. The observed and theoretical mass values of the peptides are shown in Table I. The observed mass value ($[M + H]^+$) of the free unknown amino acid isolated from the egg jelly was 244.1, which was consistent with the theoretical value (244.0) of Br-Phe.

Determination of the Isomeric Structure. In order to determine the isomeric structure, we synthesized three isomers (ortho, meta, and para) of Br-Phe and analyzed them with an amino acid analyzer. The three synthetic isomers of Br-Phe were eluted separately from each other. The retention time of free Br-Phe or SAP-derived Br-Phe corresponded to that of a synthetic *o*-Br-Phe (Figure 2).

Br-Phe from the acid hydrolysate of peptide TG-1 was isolated by RP-HPLC with use of Program IV. The synthetic *o*-Br-Phe was eluted separately from the other isomers by RP-HPLC with use of LiChrosorb RP-18 column. When analyzed under the same conditions, the Br-Phe isolated from the peptide or free Br-Phe was eluted from the column at the same retention time as the synthetic *o*-Br-Phe (Figure 3).

To confirm the isomeric structure, we analyzed Br-Phe isolated from the peptide, free Br-Phe, and an intact peptide TG-1 by ^1H NMR spectroscopy. In the aromatic region of the spectra, four sets of proton signals, which were identical with those of the synthetic *o*-Br-Phe, were observed (Figure 4, Table II). From these results, we concluded that the unknown amino acid was *o*-Br-Phe.

Determination of the Enantiomeric Structure. To determine its enantiomeric structure (D- or L-isomer), we parpared the FDAA derivative of Br-Phe isolated from peptide TG-1 and free Br-Phe and analyzed it by RP-HPLC with use of a Unisil Q C-18 column. The derivatives were eluted from the column at the same retention time as that of the synthetic *o*-Br-L-Phe derivative (Figure 5).

m-Bromophenylalanine-Containing SAP. An SAP (TG-9) that contains another unknown amino acid was also purified from *T. gratilla* egg jelly in a final amount of 21.8 nmol by RP-HPLC with use of Programs I, II, III, IV, V, and VI. Although the peptide was hydrolyzed with constantly boiling HCl, an unknown peak that was eluted at the same retention time as that of Trp was detected in the amino acid analysis (Figure 6B). Figure 1B shows a FAB mass spectrum of the peptide in the range from 850 to 1050 atomic mass units. The observed mass value (970.2) of peptide TG-9 was not consistent with the theoretical value (931.4) calculated from the amino acid composition of Asp(1), Asn(1), Gly(5), Val(1), and Leu(1) with one residue of Trp, and the isotopic ion distribution indicated the presence of a bromine atom in the peptide. This result suggests that the unknown amino acid with the same retention time as that of Trp is derived from a brominated compound. For identification of this unknown amino acid, peptide TG-9 was submitted to accurate mass measurement. The observed mass value (970.326) and the isotopic ion distribution of the peptide were consistent with the theoretical value (970.327) and the isotopic distribution calculated

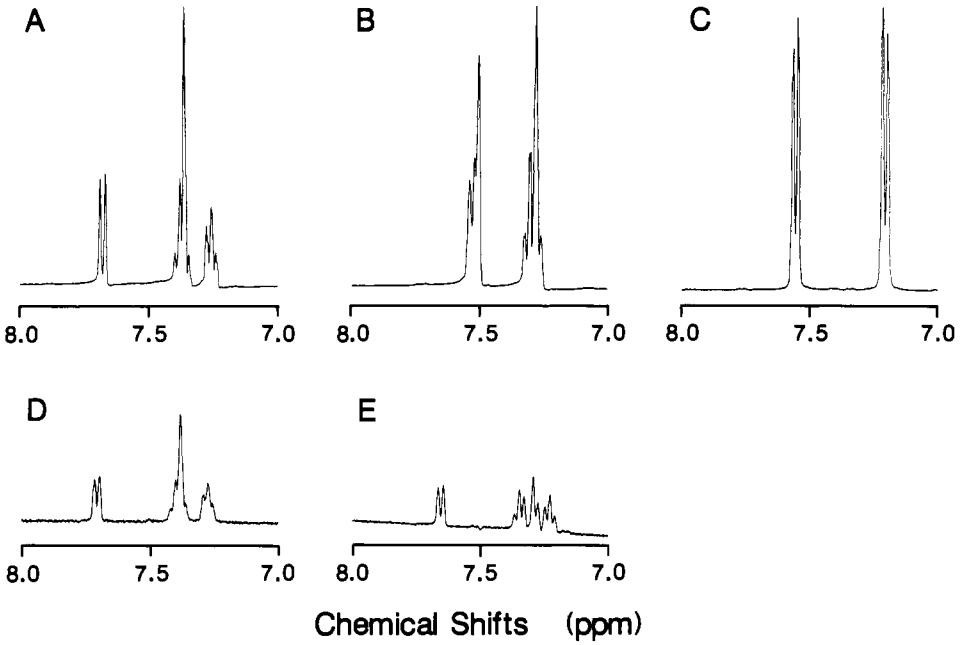

FIGURE 4: Aromatic region of the 400-MHz ^{1}H NMR spectra of the three synthetic isomers, ortho (A), meta (B), and para (C), of Br-Phe, free Br-Phe isolated from the egg jelly (D), and the intact peptide TG-1 (E).

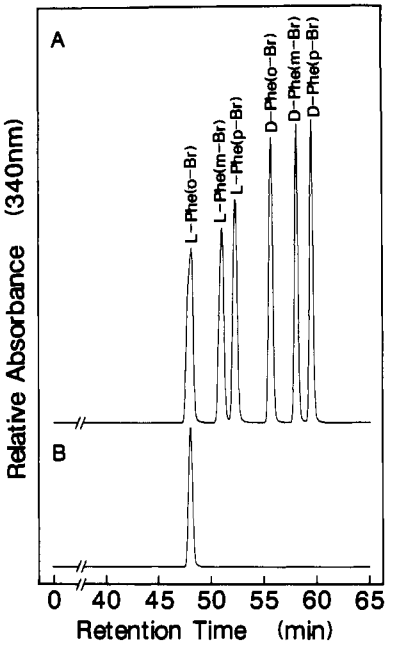

FIGURE 5: RP-HPLC profiles of the enantiomers of Br-Phe derivatized with FDAA: (A) The three synthetic isomers of Br-Phe; (B) free Br-Phe isolated from the egg jelly.

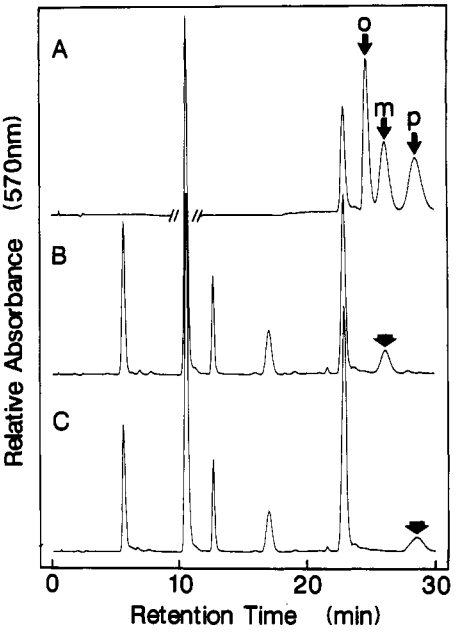

FIGURE 6: Chromatograms of amino acid analyses. Chromatogam A shows the three synthetic isomers of Br-Phe. The retention times were 24.70 (ortho), 26.14 (meta), and 28.54 min (para). Hydrolysates of peptide TG-9 and TG-10 are represented in chromatograms B and C, respectively. An unusual peak indicated by an arrow on chromatogram B or C corresponds to *m*- or *p*-Br-Phe, respectively.

from the amino acid composition of Asp(1), Asn(1), Gly(5), Val(1), Leu(1), and Br-Phe(1). In order to determine an isomeric structure of the Br-Phe, we compared three synthetic isomers of Br-Phe with the acid hydrolysate of the peptide by amino acid analysis. The retention time of the Br-Phe from peptide TG-9 was identical with that of a synthetic *m*-Br-Phe (Figure 6A,B). The molar ratio of the peak to Val was calculated to be 1.10 on the basis of color development of the synthetic *m*-Br-Phe (Table I). From these results, we concluded that peptide TG-9 contains one *m*-bromophenylalanine residue.

p-Bromophenylalanine-Containing SAP. We purified one more SAP (TG-10), which contains another unknown amino acid, in a final amount of 53.3 nmol by RP-HPLC with use of the same programs for peptide TG-9. An unusual ninhydrin-positive peak was eluted at the position just after Arg in the amino acid analysis of peptide TG-10 (Figure 6C). In order to identify the unknown amino acid, a FAB mass spectrometric method was applied to the peptide. Peptide TG-10 gave an intense signal at m/z = 969.1 (Figure 1C). The observed mass value and the isotopic ion distribution were nearly identical with the theoretical value (969.3) and the ion distribution calculated from the amino acid composition of Asn(2), Gly(5), Val(1), Leu(1), and Br-Phe(1). In the accurate mass measurement of the peptide, the observed mass value (969.343) was the same as the theoretical value. Moreover, the retention time of the unknown peak was identical with that of a synthetic *p*-Br-Phe in amino acid analysis

Table III: Amino Acid Sequences of Bromophenylalanine-Containing Sperm-Activating Peptides

TG-1	Gly-Phe(*o*-Br)-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly	([Phe(*o*-Br)^2]SAP-I)
TG-2	Gly-Phe(*o*-Br)-Asn-Leu-Asn-Gly-Gly-Gly-Val-Gly	([Phe(*o*-Br)^2,Asn^3]SAP-I)
TG-3	Gly-Phe(*o*-Br)-Asp-Leu-Asp-Gly-Gly-Gly-Val-Gly	([Phe(*o*-Br)^2,Asp^5]SAP-I)
TG-4	Gly-Phe(*o*-Br)-Ser-Ile-Gly-Gly-Gly-Gly-Val-Gly	([Phe(*o*-Br)^2,Ser^3,Ile^4,Gly^5]SAP-I)
TG-5	Gly-Phe(*o*-Br)-Ser-Leu-Ser-Gly-Gly-Gly-Val-Gly	([Phe(*o*-Br)^2,Ser^{3,5}]SAP-I)
TG-6	Gly-Phe(*o*-Br)-Asp-Leu-Gly-Gly-Gly-Gly-Val-Gly	([Phe(*o*-Br)^2,Asp^3,Gly^5]SAP-I)
TG-7	Gly-Phe(*o*-Br)-Ser-Leu-Gly-Gly-Gly-Gly-Val-Gly	([Phe(*o*-Br)^2,Ser^3,Ile^4,Gly^5]SAP-I)
TG-8	Gly-Phe(*o*-Br)-Gly-Leu-Gly-Gly-Gly-Gly-Val-Gly	([Phe(*o*-Br)^2,Gly^{3,5}]SAP-I)
TG-9	Gly-Phe(*m*-Br)-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly	([Phe(*m*-Br)^2]SAP-I)
TG-10	Gly-Phe(*p*-Br)-Asn-Leu-Asn-Gly-Gly-Gly-Val-Gly	([Phe(*p*-Br)^2,Asn^3]SAP-I)

(Figure 6A,C). From these results, we concluded that the amino acid composition of peptide TG-10 was Asn(2), Gly(5), Val(1), Leu(1), and *p*-Br-Phe(1) (Table I).

Sequence Determination. To determine the amino acid sequences of various Br-Phe-containing SAPs, a collision-induced dissociation (CID) method in FAB mass spectrometry was applied to the peptides. Then, a CID spectrum of the peptides was obtained by using a *B/E*-linked scanning method, showing the type Y_n and B_n series of sequence ions from the N- and C-terminus, respectively (Roepstorff & Fohlman, 1984). N- or C-terminal sequences were determined by considering the mass differences among Y_n or B_n sequence ions in series, respectively. As shown in Table III, the sequences of the peptides are similar to the seuqence of SAP-I.

Respiration-Stimulating Activity. The brominated peptides purified here were quite potent stimulators for respiration of *H. pulcherrimus* spermatozoa. The ortho-brominated peptides (TG-1–TG-8) stimulated respiration of spermatozoa half-maximally at concentrations between 27 and 81 pM. Those for meta- and para-brominated peptides were 51 and 126 pM, respectively.

Proportion of Bromination of the SAPs. In different and scale-down (1/50) purifications using the same procedures as described in Experimental Procedures, we isolated two brominated peptides ([*o*-Br-Phe^2]SAP-I, 8.2 nmol, and [*o*-Br-Phe^2,Asn^3]SAP-I, 2.6 nmol) and two corresponding non-brominated peptides (SAP-I, 14.1 nmol, and [Asn^3]SAP-I, 4.6 nmol). From the results, the proportion values of the ortho bromination of SAP-I and [Asn^3]SAP-I are calculated to be 1.00:0.58 and 1.00:0.57 (non-brominated:ortho-brominated), respectively.

Discussion

In the present study, we isolated eight SAPs (TG-1–TG-8) that contain *o*-bromo-L-phenylalanine and free *o*-bromo-L-phenylalanine from the egg jelly of the sea urchin *T. gratilla*. In addition to *o*-bromo-L-phenylalanine, we discovered both *m*- and *p*-bromophenylalanines in the peptides TG-9 and TG-10 isolated from the egg jelly of the same species. A number of brominated amino acids have been isolated from marine invertebrates; 3-bromotyrosine, 3,5-dibromotyrosine, and 3-chloro-5-bromotyrosine from *Limulus polyphemus* (Welinder, 1972), 3-bromo-dityrosine, 3,3′-dibromodityrosine, and 3-bromotrityrosine from *Cancer pagurus* (Welinder, 1976), 6-bromotryptophan from *Mytilus edulis* (Waite & Andersen, 1980), and L-6-bromohypaphorine from *Pachymatisma johnstone* (Hunt, 1985). Although *p*-bromophenylalanine was identified in theonellamide F, an antifungal bicyclic peptide from the marine sponge *Theonella* sp. (Matsunaga et al., 1989), the present study is the first report of the presence of *o*- and *m*-bromophenylalanines in natural materials. It is also a demonstration of bromophenylalanine in SAPs.

In the past decade, we have isolated 23 SAPs that possessed phenylalanine or tyrosine using identical procedures as described here; none of them had a bromophenylalanine or bromotyrosine. In the present study, however, we paid precautions to prevent possible chemical production of bromophenylalanine during extraction, purification, and acid hydrolysis of SAPs. Therefore, we think that there is little possibility that bromophenylalanines were generated artificially in the processes of extraction, purification, and acid hydrolysis of the peptides. Non-brominated SAPs stimulated respiration of sea urchin spermatozoa half-maximally at about 14–50 pM (Yoshino et al., 1989). The corresponding Br-Phe-containing SAP-I and SAP-I derivatives isolated in the present study stimulated sperm respiration half-maximally at the concentrations between 27 and 126 pM. These values are comparable to those with non-brominated SAP-I and SAP-I derivatives. Studies on the structure–activity relationship of SAP-I demonstrated that the aromatic residue at position 2 is essential for the activity (Nomura & Isaka, 1985; Nomura, 1987). In the present study, ortho-, meta-, or para-bromo group substitution on the phenylalanine residue retained almost full respiration-stimulating activity toward sea urchin spermatozoa. At present, we do not know the biological significance of bromination of SAPs.

In a previous paper (Yoshino et al., 1989), we reported the isolation of non-brominated SAPs whose amino acid sequences were identical with the brominated SAPs reported here. Therefore, brominated SAPs may be generated by bromination of non-brominated SAPs. Since iodination of tyrosine residues in thyroglobulin is catalyzed by thyroid peroxidase (Neary et al., 1984), the bromination of a phenylalanine residue in SAPs may be carried out by a bromoperoxidase, which has not been isolated from sea urchins but has been isolated from some species of marine algae (Manthey et al., 1984; Wever et al., 1985; Krenn et al., 1989). Most of the halogenated amino acids found in proteins are components of skeletal materials of marine invertebrates (Hunt, 1984). Many studies on the halogenation of invertebrate scleroprotein suggest that the process is a secondary consequence of a cross-linking reaction by phenol oxidase (Welinder, 1972; Hunt, 1984).

The results from small-scale purification in the present study indicated that the proportion values of ortho bromination in two major peptides (SAP-I and [Asn^3]SAP-I) were about 60%. With consideration of the final amounts of the peptides obtained in large-scale purification and above the proportion value, the proportion of the bromination of SAP-I is estimated to be 5:3:0.3 (non-brominated:ortho-brominated:meta-brominated). Similarly, as to the bromination of [Asn^3]SAP-I, the proportion is calculated to be 5:3:3 (non-brominated:ortho-brominated:para-brominated). These high proportions of bromination of the egg peptides in *T. gratilla* suggest that the bromination of the peptides does not artificially occur during extraction, purification, and acid hydrolysis of the peptides and that the sea urchin has a specific bromination system in the ovary.

Ramarao et al. (1990) have isolated complementary DNA encoding SAP-I, demonstrating that a single mRNA encodes

multiple copies of SAP-I and its derivatives. Therefore, the bromination of SAPs would be carried out posttranslationally. It is well-known that the enzymatic electrophilic bromination reaction of the phenylalanyl aromatic ring is in the ortho–para orientation. It has been reported that posttranslational modification of a given amino acid can occur at any stage of protein synthesis after the formation of the aminoacyl-tRNA (Wold, 1981). Thus, substitution of the bromo group at the ortho or para position on the phenylalanyl aromatic ring could occur at any process in posttranslational modification.

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Registry No. *o*-Br-Phe, 42538-40-9; *m*-Br-Phe, 82311-69-1; *p*-Br-Phe, 24250-84-8; TG-1, 133872-39-6; TG-2, 133886-92-7; TG-3, 133872-40-9; TG-4, 133872-41-0; TG-5, 133872-42-1; TG-6, 133872-43-2; TG-7, 133872-44-3; TG-8, 133872-45-4; TG-9, 133872-46-5; TG-10, 133872-47-6.

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