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Presence of 3,4-dihydroxyphenylalanine-containing peptides in hemocytes of the ascidian, *Halocynthia roretzi*

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Summary. Five 3,4-dihydroxyphenylalanine (DOPA)-containing peptides have been isolated from hemocytes of the ascidian, *Halocynthia roretzi*. Three of them were composed of DOPA, proline, phenylalanine, histidine and arginine in different ratios, while the other two contained only DOPA and an unidentified amino acid. DOPA-containing peptides were found to exist in only one type of hemocyte.
Key words. 3,4-dihydroxyphenylalanine; DOPA-containing peptide; hemocyte; ascidian; boronate immobilized column.

Two 3,4-dihydroxyphenylalanine (DOPA)-containing proteins have been isolated from tissues and hemocytes of invertebrates [1-4]. One protein with a molecular weight of 130,000, isolated from the phenol gland of the marine mussel, *Mytilus edulis*, contains 11% DOPA and a large amount of hydroxyproline (13%)[1,2]. The other is named ferreascidin; this has been isolated from hemocytes of the stolidobranch ascidian, *Pyura stolonifera*, and has 17% DOPA and a large amount of tyrosine (42%)[3,4]. The molecular weight is about 10,000. Furthermore, modified tripeptides containing DOPA and/or hydroxy-DOPA and compounds related to them have been isolated from hemocytes of the phlebobranch ascidian, *Ascidia nigra*, and the stolidobranch ascidian, *Molgula manhattensis*, as reducing blood pigments[5,6]. They are designated as tunichromes.

In a previous communication[7], we have reported the presence of two DOPA-containing tetrapeptide-like substances named halocyamine A and B in the acetone-extract of hemocytes of the stolidobranch ascidian, *Halocynthia roretzi*. Halocyamine A consists of DOPA, histidine, glycine, and a tryptophan derivative, while B consists of DOPA, histidine, threonine, and the same tryptophan derivative. They are present in only one type of hemocyte and show antibacterial activity. Thus, we have proposed that they may play important roles in the defense mechanisms of *H. roretzi*.

The demonstrations[3,4] showing the presence of DOPA-containing proteins in hemocytes of the ascidian of the stolidobranch led us to hypothesize that halocyamines would be derived from putative DOPA-containing precursors present in *H. roretzi* hemocytes because *H. roretzi* belongs to the same suborder, the stolidobranch. In the course of studies undertaken to test the hypothesis, we found DOPA-containing peptides other than halocyamines in the aqueous extract of the hemocytes. In this paper, we describe the isolation and characterization of DOPA-containing peptides of *H. roretzi* hemocytes.

Materials and methods

Solitary ascidians *H. roretzi*, type C, were harvested in Mutsu Bay, Japan. Hemocytes were collected as described previously[7] and were frozen at − 20 °C until used.

The measurement of DOPA was carried out according to a modification of the fluorometric assay described previously[8]. To 50 µl of a solution of DOPA-containing peptide was added 350 µl of 10 mM phosphate buffer, pH 6.5, containing 0.0025% zinc sulfate. After mixing, 5 µl of 0.25% potassium ferricyanide was added and then the mixture was shaken for 2 min (oxidation step). The oxidation reaction was stopped by addition of 50 µl of a mixture consisting of 5 M NaOH and 2% ascorbic acid in a ratio of 9:1 (v/v). Five minutes later the fluorescence intensity derived from the reaction product, DOPAquinone, was measured with excitation at 360 nm and emission at 490 nm.

DOPA-containing peptides were isolated from *H. roretzi* hemocytes as follows: Frozen hemocytes (2.5 g wet weight) were thawed and homogenized with 7 ml of 0.1 M sodium acetate, pH 5.0, containing 10 mM EDTA and 5 mM ascorbic acid using a Teflon homogenizer (1300 rpm, 5 strokes). The homogenate was centrifuged (9000 × g, 15 min) and the resulting supernatant was used as a crude extract of the DOPA-containing peptides. The extract (6 ml) was applied to a column (1.4 ± 54 cm) of Sephadex G-50 previously equilibrated with 10 mM sodium acetate, pH 5.0, containing 20 mM NaCl, 0.1 mM EDTA, and 0.1 mM ascorbic acid, and the column was developed with the same equilibrating buffer. DOPA was detected in fractions eluted at about 79 ml as a single peak with shoulders.

Fractions (1.5 ml) where DOPA was detected were pooled and subjected to high-performance liquid chromatography (HPLC) on a column (7.5 × 75 mm) of boronate-5PW (TOSOH, Japan) which had been previously equilibrated with buffer A (10 mM sodium acetate, pH 5.0, containing 0.1 mM EDTA, 20 mM NaCl, and 0.01 mM ascorbic acid). After the column had been successively washed with buffer A, buffer B (10 mM Tris-HCl, pH 7.5, containing 0.1 mM EDTA, 20 mM NaCl, 1 mM sodium borate, 0.2 M sorbitol, and 0.01 mM ascorbic acid), and again buffer A, DOPA-containing peptides were eluted with buffer C (10 mM sodium acetate, pH 3.0, containing 0.1 mM EDTA, 20 mM NaCl, and 0.01 mM ascorbic acid). Buffer C-eluted fractions (0.2 ml) were pooled and subjected to HPLC using a reversed-phase column (0.6 × 15 cm) of YMC pack A-312 (Yamamura Chem. Co., Japan) (fig. 1). The column had been previously equilibrated with 0.05% trifluoroacetic acid (TFA). Elution was performed with a 30-min linear gradient of 0–40% acetonitrile in 0.05% TFA at a flow rate of 1 ml/min. The absorbance at 280 nm was monitored. Six major peaks (a–f) were detected and pooled separately.

The amino acid composition was analyzed with a Hitachi 835 amino acid analyzer after hydrolysis with 6 M HCl containing 1% phenol at 110 °C for 24 h. Amino acids were detected by reaction with ninhydrin. Alternatively, phenylthiocarbamoyl-amino acids derived by modification with phenylisothiocyanate were separated by reversed-phase HPLC on a PTC-C18 column (2.1 × 220 mm, Applied Biosystems) and their contents were determined by using yields of standard amino acid derivatives.

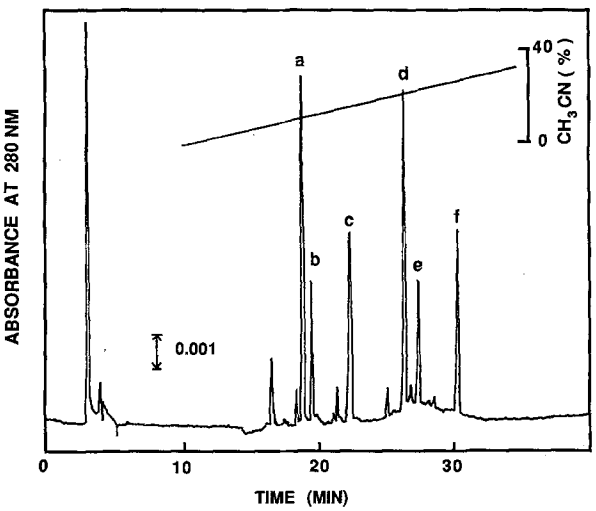

Figure 1. Reversed-phase HPLC of DOPA-containing peptides from hemocytes of *H. roretzi*. An aliquot in a volume of 5 µl of the acetate-eluted fraction obtained from the boronate-5PW column was injected into a YMC pack A-312 column (0.6 × 15 cm) that had been previously equilibrated with 0.05% trifluoroacetic acid. Elution was performed with a 30-mm linear gradient of 0–40% acetonitrile in 0.05% trifluoroacetic acid at a flow rate of 1 ml/min. The absorbance at 280 nm was monitored.

SDS-polyacrylamide gel electrophoresis was performed in a slab gel containing 15% polyacrylamide according to the method of Laemmli[9]. Protein was stained with Commassie Brilliant Blue.

Hemocytes of *H. roretzi* were separated into two parts, hemocytes located in the upper percoll density gradient (upper fraction) and ones recovered in the precipitate fraction (bottom fraction), by percoll density gradient centrifugation as described previously[7].

Results and discussion

DOPA-containing peptides were isolated from hemocytes of *H. roretzi* by gel filtration on Sephadex G-50, and HPLC using boronate-5PW and reversed-phase columns. They were detected in the supernatant obtained by centrifugation of hemocyte homogenate and appeared in low molecular-weight fractions as a single peak with shoulders in gel filtration. Since boronate-immobilized adsorbent has been reported to be effective in separating catechol compounds[3,4], we used boronate-5PW as an adsorbent to trap DOPA-containing peptides. DOPA-containing peptides were efficiently adsorbed on this column and eluted with a yield of 44% by lowering the pH to 3.0. Free DOPA, catechols, and sugars have been reported to be eluted from the column with the sorbitol-including buffer (pH 7.5)[3,4], which is buffer B used in the washing step in this study. DOPA-containing peptides obtained from the boronate-5PW column have broad bands in the region with a molecular weight less than

6500 on SDS-polyacrylamide gel electrophoresis (data not shown). So we employed reversed-phase HPLC as the final purification step to separate DOPA-containing peptides from one another. The peak correspondent to that of free L-DOPA which appeared at the retention time of 17 min was not detected in the chromatogram shown in figure 1. Although six major peaks were separated by the HPLC, detected by measuring absorbance at 280 nm (fig. 1), the results of amino acid composition analyses (table) showed that five peptides contained DOPA. Peptide C was found not to have DOPA. Although L-DOPA is found at the same position as that of leucine in amino acid analysis detected by ninhydrin, they can be separated from each other by reversed-phase HPLC on a PTC-C18 column after modification with phenylisothiocyanate. A leucine-derivative (phenylthiocarbamoylleucine) could not be detected in any of the five DOPA-containing peptides nor peptide C. So we assigned an amino acid, eluted at the same position in amino acid analysis as of leucine, as DOPA, and determined its amount in amino acid analysis by using the color yield of standard DOPA. Phenylthiocarbamoyl-DOPA separated by the reversed-phase HPLC in either peptide was found to be detected in the same molar ratio as that shown in the table.

The five DOPA-containing peptides can be classified into two groups; peptides a and b consisted of DOPA and an unidentified amino acid (the latter amino acid was eluted as a single sharp peak but corresponded with none of the standard amino acids), while peptides d, e, and f contained DOPA, proline, phenylalanine, histidine, and arginine. Since other amino acids including tyrosine and tryptophan were not detected in either of the peptides (tryptophan was undetectable even when the peptide was hydrolyzed with 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole[10]), it can be concluded that neither of the peptides was correspondent with or derived from the precursors of halocyamines. It is necessary to search for high molecular-weight DOPA-containing polypeptides present in hemocytes of *H. roretzi* in small amounts in order to confirm the hypothesis that halocyamines are derived from precursors biosynthesized in the hemocytes. In any case, it is of great interest that DOPA-containing small peptides showing simple amino acid compositions are present in *H. roretzi* hemocytes.

Amino acid compositions of DOPA-containing peptides

Amino acid*	Mole % a	b	c	d	e	f
Proline	0.0	0.0	0.0	20.0	19.6	20.7
Phenylalanine	0.0	0.0	52.3	19.7	19.6	19.7
Histidine	0.0	0.0	0.0	39.8	40.0	29.2
Arginine	0.0	0.0	12.6	20.0	19.3	20.1
DOPA	39.4	20.7	0.0	0.6	1.5	10.3
Unknown	60.6	79.3	35.1	0.0	0.0	0.0
Total	100.0	100.0	100.0	100.1	100.0	100.0

*Other amino acids were undetectable.

Next, we examined the distribution of DOPA-containing peptides among *H. roretzi* hemocytes. The hemocytes were separated into two groups (upper and bottom fractions) using percoll gradient centrifugation. The content of DOPA in the aqueous extract of the bottom fraction was more than 100-fold higher than that in the upper fraction (fig. 2). The results (fig. 3) on boronate-5PW HPLC of the aqueous extracts of both the upper and bottom fractions indicate that the content of DOPA-containing peptides eluted with acetate buffer (buffer C) in the latter fraction was about 10-fold higher than that in the former. It has been shown that only one type of hemocyte containing halocyamines is present in the bottom fraction[7]. In connection with the above hypothesis,

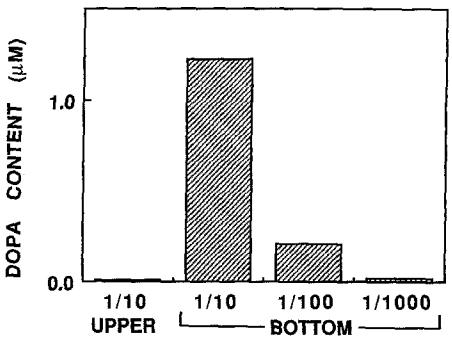

Figure 2. DOPA contents in the aqueous extracts of upper and bottom fractions of hemocytes of *H. roretzi*. Hemocytes obtained from an ascidian were separated by percoll density gradient centrifugation. The hemocytes present in the upper and bottom fractions were separately washed by centrifugation and extracted with the same volume (1 ml) of the homogenizing buffer. DOPA content in each of the extracts diluted 10-fold serially was measured. The number in the horizontal axis indicates the dilution of each extract.

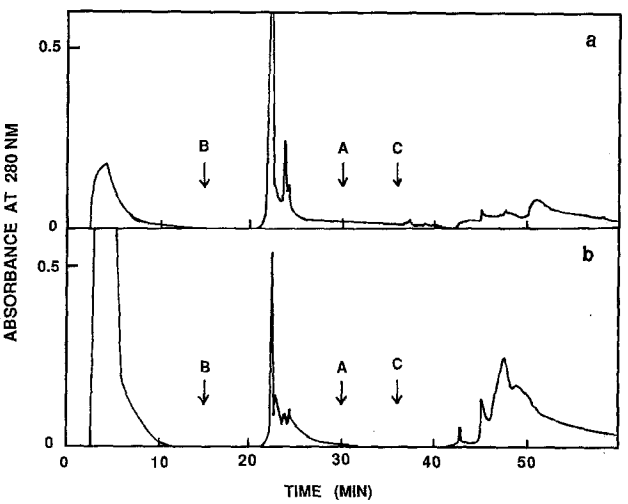

Figure 3. Boronate-5PW HPLC of aqueous extracts of (*a*) upper and (*b*) bottom fractions of hemocytes of *H. roretzi*. The extract (2.5 ml) of each fraction was applied to a boronate-5PW column (7.5 × 75 mm) which had been previously equilibrated with buffer A. After the column had been washed with buffer A, buffer B, and again buffer A, DOPA-containing peptides were eluted with buffer C at a flow rate of 1 ml/min. The absorbance at 280 nm was monitored. The compositions of buffer A, B, and C were described in 'Materials and methods'.

it should be noted that DOPA-containing peptides were co-localized with halocyamines in the same hemocytes. Like DOPA-containing peptides and halocyamines of *H. roretzi*, tunichromes of *A. nigra* and *A. ceratodes* have been reported to be mainly localized in one type of their hemocytes [11].

Several biological roles have been proposed for DOPA-containing proteins or peptides. DOPA-containing protein of the marine mussel, *Mytilus edulis*, functions as an adhesive protein through its many repeating DOPA-containing sequences [1, 2]. Ferriascidin of the ascidian, *P. stolonifera*, is assumed to be an iron-binding protein [3, 4]. Tunichromes of the ascidians are potent reductants and are proposed as vanadium-binding and iron-binding compounds in the vanadium-sequestering ascidian, *A. nigra*, and the iron-sequestering one, *M. manhattensis*, respectively [5, 6]. Halocyamines of the ascidian, *H. roretzi*, show antibacterial and cytotoxic activity [7]. We made a preliminary examination of the antibacterial activity of DOPA-containing peptides isolated in this study against *Bacillus subtilis*, by the paper disc method, but could not detect any. It has been reported that vanadium is accumulated at a high concentration in hemocytes of ascidians of the phlebobranch, whereas hemocytes of *H. roretzi* of the stolidobranch contain iron in an amount higher than that of vanadium [12, 13]. Our preliminary experiments showed that halocyamines can reduce ferric iron to ferrous iron. Thus, DOPA-containing peptides including halocyamines isolated from *H. roretzi* hemocytes may function in binding iron, like the ferriascidin of *P. stolonifera* hemocytes and tunichromes of *M. manhattensis* hemocytes.

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Pentobarbital-induced phase shifts of circadian rhythms of locomotor activity are not mediated through stimulated activity in mice

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Summary. The possibility that phase shifts of circadian rhythms of locomotor activity induced by pentobarbital injections are mediated through hyperactivity after recovery from the sedative condition was tested in DBA/2 mice. The mice were restrained for 3 h in a tube immediately after injections of pentobarbital at either CT 9 or CT 0. The results indicated that immobilization did not block the phase shifts, suggesting that pentobarbital-induced phase shifts are not due to increasing the level of activity.
Key words. Circadian rhythms; pentobarbital; phase shifts; mice; immobilization; hyperactivity.

It has been generally accepted that the circadian clock is independent of changes in the external and internal environment and the clock itself is not affected by clock-controlled events such as changes of the level of locomotor activity. However, recent studies in hamsters have indicated that in certain circumstances the clock may be susceptible to feedback from overt activity. Mrosovsky's group has clearly indicated that induced wheel-running brought about by cage-changing and social interactions can induce phase shifts in the circadian clock of the hamster [1, 2]. These findings raised the possibility that the phase-shifting effects reported for some drugs may be mediated by changes of behavioral events, not by direct effects of the drugs on the circadian system. In fact, phase shifts induced by triazolam, which causes hyperactivity in the hamster, are totally blocked by immobilization of the animal during treatment [3].

We have reported that γ-aminobutyric acid (GABA)-active drugs including triazolam, pentobarbital and muscimol induce phase-dependent phase shifts of the circadi-