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Radiochromatographic assay of metabolites of the oostatic peptide labeled in different positions of the peptide chain☆

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Abstract

Reversed-phase high-performance liquid radio-chromatography (radio-HPLC) was set up to detect the time course of labeled degradation product formation of the pentapeptide H-Tyr-Asp-Pro-Ala-Pro-OH (5P), which has oostatic effects in different insect species. The detection limit of the system was in the range of 80–150 Bq. To follow formation of the degradation products, three amino acid residues in 5P were independently tritiated: Tyr[1], Pro[3] and Pro[5]. Each of the three tritiated peptides was analyzed after incubation with fresh hemolymph or ovaries of *Neobellieria bullata*. In the incubation mixture, free terminal amino acids and shortened sequences of 5P were identified. A metabolite of tyrosine represented the only exception; it was finally identified as water using degradation of [^{3}H]Tyr by tyrosinase. Metabolic degradation of [^{3}H]Tyr-5P was found to be considerably quicker than that of H-[^{3}H]Tyr-Asp-Pro-Ala-OH (4P). The degradation of 5P was considerably slower in ovaries in comparison to hemolymph.

Keywords: Peptide synthesis; Radiolabeling; Radio-HPLC; Course of degradation; Tyrosine metabolism

1. Introduction

In comparison to pesticides [1], oligopeptides may have advantages in insect pest control. Apart from easier synthesis and solubility in water, they cause considerably less or no pollution of the subsurface environment [2].

Our investigation proved the deteriorating effect of the decapeptide H-Tyr-Asp-Pro-Ala-Pro$_6$-OH (10P) [3] isolated from the mosquito *Aedes aegypti* by Borovsky [4] on ovarian development (i.e. oostatic effect) of species Diptera, Orthoptera and Hemiptera [5]. The highest effect was found for C-terminally truncated analogues H-Tyr-Asp-Pro-Ala-Pro-OH (5P) and H-Tyr-Asp-Pro-Ala-OH (4P). It was shown previously [3] that after their application the hatchability was lower compared to controls and the development of egg chambers of the second gonadotrophic cycle was pathologically modified. The nuclei of follicular cells formed a multinuclear layer, which proliferated towards the inner part of the egg chamber. Such eggs were not able to complete their development and were later resorbed. To enrich our knowledge about the fate of these oostatic peptides after application to *Neobellieria bullata* (Diptera), metabolic degradation of 5P was analyzed in this paper.

A highly sensitive radio-HPLC [2,6,7] was selected to quantify small amounts of 5P metabolites in the presence of a relatively high background of other organic compounds. Three

Abbreviations: AAA, amino acid analysis; ACN, acetonitrile; AcOH, acetic acid; DCM, dichloromethane; DIC, *N*,*N*-diisopropylcarbodiimide; DMA, dimethylacetamide; DMF, *N*,*N*-dimethylformamide; FAB MS, fast atom bombardment mass spectrometry; Fmoc, [(fluoren-1-yl-methoxy]carbonyl; HOBt, 1-hydroxybenzotriazole; *t*Bu, tert-butyl; TFA, trifluoroacetic acid

☆ The nomenclature and symbols of amino acids follow Recommendations of IUPAC/IUB Joint Commission on Biochemical Nomenclature (1984).

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Table 1
Radiolabeled peptides prepared for the degradation study

No.	Labeled sequence
1a	H-Asp-[^{3}H]Pro-OH
1b	H-Tyr-Asp-[^{3}H]Pro-Ala-OH
1c	H-Tyr-Asp-[^{3}H]Pro-Ala-Pro-OH
1d	H-Tyr-Asp-Pro-Ala-[^{3}H]Pro-OH
1e	H-Tyr-Asp-Pro-Ala-Pro$_5$-[^{3}H]Pro-OH
1f	H-[^{3}H]Tyr-Asp-Pro-Ala-OH
1g	H-[^{3}H]Tyr-Asp-Pro-Ala-Pro-OH

Table 2
Synthesized non-labeled peptide HPLC standards

Peptide no.	Amino acid sequence
2a	H-Asp-Pro-Ala-Pro-OH
2b	H-Pro-Ala-Pro-OH
2c	H-Ala-Pro-OH
2d	H-Tyr-Asp-Pro-OH
2e	H-Tyr-Asp-OH
2f	H-Asp-Pro-Ala-OH
2g	H-Pro-Ala-OH
2h	H-Asp-Pro-OH

radiolabeled derivatives of 5P were prepared for this study. Selective tritiation of tyrosine and proline residues in the peptide sequence made it possible to resolve the time course of production of the individual degradation products. Radio-HPLC also enabled an analysis of a tyrosine metabolite found in the mixture of degradation products.

Previously, synthesis and characterization of precursors of the tritiated peptides, i.e. 3,4-dehydroproline analogues **1a**–**1e** [8] and of the standards 4P and 5P [9] used also for tritiation **1f** and **1g**, have been described (Table 1).

In addition to the tritiated oostatic peptides, their non-labeled fragments truncated in the amino (**2a**–**2c**), carboxy (**2d**, **2e**) or both the termini (**2f**–**2h**) were synthesized as standards for HPLC study (Table 2). As standards corresponding to the carboxy- and amino-terminus, amino acids proline and tyrosine were used, respectively.

2. Materials and methods

Tyrosine: $C_9H_{11}NO_3$ (181.19), >99% (non-aqueous titration) $[\alpha]_D^{20} - 11.5^\circ$ (0.04 g/ml of 1N HCl) and proline: $C_5H_9NO_2$ (115.13), >99% (non-aqueous titration) $[\alpha]_D^{20} - 84.5$ (0.05 g/ml of water) were obtained from Fluka Chemie AG (Buchs, Switzerland). Fmoc-Asp(O*t*Bu)-OH: $C_{23}H_{25}NO_6$ (411.5), 99.8%, mp 147–148 °C, $[\alpha]_D^{20} - 23.8$ (0.01 g/ml of DMF), 0.1% D-enantiomer and Fmoc-Tyr(*t*Bu)-OH: $C_{28}H_{29}NO_5$ (459.5), 99.9%, mp 151–152 °C, $[\alpha]_D^{20} - 29.4$ (0.01 g/ml of DMF), 0.1% D-enantiomer were purchased from Senn Chemicals AG (Dielsdorf, Switzerland). Fmoc-Pro-OH: $C_{20}H_{19}NO_4$ (337.38), 99.8%, mp 109–110 °C, $[\alpha]_D^{20} - 32.2$ (0.01 g/ml of DMF), 0.1% D-enantiomer and Fmoc-Ala-OH: $C_{18}H_{17}NO_4$ (311.31), 99.8%, mp 152–153 °C, $[\alpha]_D^{20} - 18.5$ (0.01 g/ml of DMF), 0.2% D-enantiomer were prepared in our laboratory following general protocols [10]. 2-Chlorotritylchloride resin (200–400 mesh, 1% DVB, 1.3 mmol/g) was purchased from Calbiochem-Novabiochem AG (Läufelfingen, Switzerland). Enzymes L-amino acid oxidase, tyrosinase and tyrosine decarboxylase, and anisole were from Sigma–Aldrich (Germany).

Solvents were evaporated in vacuo on a rotary evaporator (bath temperature 30 °C). Electrophoreses were carried out on Whatman 3 MM paper (moist chamber, 20 V/cm, 1 h) in 1 M AcOH (pH 2.4) and in a pyridine–acetate buffer (pH 5.7) [11]. The compounds were visualized by ninhydrin. The samples for AAA were hydrolyzed with 6 M HCl containing 3% of phenol at 110 °C for 20 h. The AAA was performed on a Biochrom 20 instrument (Pharmacia, Sweden). Molecular weight of the peptides was determined using mass spectroscopy with FAB technique (Micromass, England). For HPLC, a Spectra Physics instrument with an SP 8800 pump, an SP 4290 integrator and Thermo Separation Products Spectra 100 UV detector was used. The compounds were purified by semipreparative HPLC on a 25 cm × 1 cm column, 10 μm Vydac RP-18 (The Separations Group, Hesperia CA, USA), flow rate 3 ml/min, detection at 220 nm, using a 0–100% gradient of ACN in 0.05% aqueous TFA (v/v) for 120 min. The analytical HPLC was carried out on a 15 cm × 0.4 cm column, 5 μm LiChrospher WP-300 RP-18 (Merck, Germany), flow rate 1 ml/min, detection at 220 nm, using a 0–100% gradient of ACN in 0.05% aqueous TFA (v/v) for 40 min unless otherwise stated. The temperature of the chromatographic column was ambient for all HPLC systems.

2.1. Synthesis of pentapeptide fragments

The peptides **2a**–**2c** were synthesized starting from Fmoc-Pro-O-2-chlorotrityl-polystyrene resin (1.0 g, substitution 0.3 mmol/g), which was swollen in DMF/DCM (1:1) and then deprotected by 5% piperidine in DMF for 10 min (30 ml) followed by 20% piperidine in DMF (30 ml) for 30 min to obtain H-Pro-O-2-chlorotrityl-polystyrene resin. This resin was gradually acylated with three equivalents of Fmoc-Ala-OH (0.29 g) in the dipeptide **2c**, Fmoc-Pro-OH (0.31 g) in the tripeptide **2b** and Fmoc-Asp(O*t*Bu) (0.38 g) in the tetrapeptide **2a** preparations. These acylation reactions were mediated by HOBt/DIC mixture (0.19 g, 1.4 mmol/0.21 g, 1.02 mmol) in DMF (30 ml). The progress of each coupling was monitored by ninhydrin test [12]. In each step, the cleavage of the Fmoc group was carried out by 20% piperidine in DMF (two times using 30 ml for 30 and 45 min). Finally, the TFA/anisole mixture (10 ml, 9:1) was used for the simultaneous cleavage of peptides **2a**–**2c** from the resin and the removal of tBu protecting groups when Asp and Tyr residues were presented. The tripeptide **2d** and dipeptide **2h** were prepared using the above procedure; the H-Pro-O-2-chlorotrityl-polystyrene resin was subsequently acylated with Fmoc-Asp(O*t*Bu)-OH (0.38 g) and Fmoc-Tyr(*t*Bu)-OH (0.43 g) in the dipeptide **2h** and tripeptide **2d** preparations, respectively.

The syntheses of peptides **2f** and **2g**, using the above described procedure, started with Fmoc-Ala-O-2-chlorotrityl-

polystyrene resin (0.9 g). After Fmoc deprotection, the resin was acylated with Fmoc-Pro-OH (0.31 g) (**2g**) and after that with Fmoc-Asp(O*t*Bu) (0.38 g) (**2f**). In the synthesis of dipeptide **2e**, Fmoc-Asp(O*t*Bu)-O-2-chlorotrityl-polystyrene resin (0.8 g, 0.7 mmol/g) was deprotected as described above and Fmoc-Tyr(*t*Bu)-OH (0.8 g, 3 equiv.) was coupled to it using the above described procedure. The peptides prepared were purified by semipreparative HPLC.

2.2. ^{3}H-labeling

For radiolabeling, the following two methods were used: (A) hydrogenation of double bond in dehydroproline residue with tritium gas [13] for **1a**–**1e** (Table 1) and (B) catalytic exchange of β-hydrogens for tritium in tyrosine residue [14] for **1f** and **1g** (Table 1). The method B was also used for the preparation of [^{3}H]Tyr (1.25 TBq/mmol, radiochemical purity >95%) for the study of its enzymatic degradation.

DMA as solvent was carefully purified, and all aqueous solutions were prepared from redistilled water. Non-labeled peptide (for A, 3,4-dehydroproline containing ones; for B, 4P or 5P), the catalyst PdO/$BaSO_4$ (10% Pd) and 0.3 ml of the solvent (DMA for A; 0.1 M $(NH_4)_2CO_3$ in water for B) were stirred for 1 h with tritium gas at a pressure of 80 kPa. After the solvent evaporation, the residue was suspended in 0.5 ml water and the catalyst removed by centrifugation and washed using 0.5 ml of water. Combined supernatants were freeze-dried. The lyophilisate *w*as purified by HPLC on a 25 cm × 0.4 cm column, 5 μm LiChrospher WP-300 RP-18 (Merck, Germany), flow rate 0.6 ml/min, isocratic elution by a mixture of ACN in 0.05% aqueous TFA (ACN: 12% for **1e**; 9% for **1c**, **1d** and **1g**; 7.3% for **1b** and **1f**; 0% for **1a**). UV spectrophotometry (275 nm) was used for the determination of the mass of the products except for **1a** for which HPLC was applied. The conditions of labeling are summarized in Table 3.

The highest stability of stock solutions of non-labeled standards (1 or 5 mg/ml) as well as solutions of labeled peptides was achieved by storage in water solution with 10% ethanol at liquid nitrogen temperature. For the check of purity after labeling and/or before each application, the following procedure was used. Seven spiking solutions of [^{3}H]Tyr^{1}-5P (from 37 to 370 kBq/ml) were prepared by appropriate dilution of the stock solution using re-destilled water. The calibration solutions for the testing of linearity were prepared by addition of 100 μl of [^{3}H]Tyr^{1}-5P spiking solution to 1 ml of hemolymph-free physiological solution.

2.3. Radio-HPLC analysis

Radio-HPLC measurements were performed on a liquid chromatograph (Waters, Model 490 E, USA) with manual injector and a programmable UV detector connected on-line to a radiometric flow-through detector (Beckman, Model 171) used together with a solvent delivery module for scintillator pumping. The stainless steel analytical column 25 cm × 0.4 cm LiChroCART, packed with LiChrospher WP-300, with a particle size 5 μm (Merck, Germany) was used. The column was protected with a 4 mm × 4 mm guard column packed with LiChrospher 100 RP-18, particle size 5 μm. The mobile phase was composed of two phases: the aqueous phase (0.035% TFA in redistilled water) and the organic phase (0.05% TFA in ACN). After passing through the UV detector, the eluent was continuously mixed with the liquid scintillator Ready Safe (Beckman, USA) in a ratio of 1:2.5 (v/v) in an on-line connected mixer. The mixture runs through a 500 μl detection cell. The threshold of radiometric detector was set at 0.02%. The UV detector was set at 230 nm, 0.05 AUFS. Separation of analyzed peptides and their metabolites was performed at ambient temperature. A number of gradient and isocratic procedures were tested. The best separation was achieved using 30 min linear gradient from 0% to 30% organic phase, followed by a 5 min gradient from 30% to 100% organic phase using a flow rate of 0.8 ml/min and continuous degassing with helium. After a further 10 min flushing of the column with 100% ACN, the program was changed to initial conditions in 5 min and the column was re-equilibrated for next 20 min at isocratic run. One microliter samples were injected. The area of each peak was evaluated as the ratio of its counting rate (cpm) to the totally measured cpm in all peaks of the appropriate radiochromatogram (relative concentration c_{rel} in percentage). The radiochemical stability of tritiated pentapeptide standard was checked before each experiment.

Table 3
Conditions of the radiolabeling and characterization of resulting peptides

Peptide	Precur.[a] quant. (mg)	Catal.[b] quant. (mg)	$^{3}H_2$ (%)	Yield		Spec. activity (TBq/mmol)	Radiochr. purity (%)
				mg	GBq		
1a	0.9	2.5	100	0.27	2.02	1.70	>97
1b	0.9	7.1	100	0.52	2.02	1.80	>95
1c	0.4	4.1	50	0.17	0.44	1.44	>98
1d	1.2	6.2	100	0.67	2.61	2.17	>97
1e	1.3	8.5	85	0.60	1.08	1.91	>95
1f	0.3	3.4	50	0.11	0.29	1.22	>98
1g	0.4	8.7	50	0.18	0.46	1.42	>98

[a] Precur. means the appropriate non-active precursor.
[b] Catal. means PdO/$BaSO_4$ catalysator.

2.4. Insect

Flesh fly *N. bullata* (Diptera) was used as a model insect. Female flies (approximate weight 90 mg), raised at 25 °C, with 12-h light:12-h dark cycle and 60% RH, were used in the experiments. Larvae were raised on beef liver covered with sawdust (pupariation medium). Pupae were separated from the medium and placed into nylon cages before emergence. Flies were fed on sugar until experiment.

2.5. Preparation of insect hemolymph and ovaries

Hemolymph from 3 to 4-day-old females was collected by means of a 5 μl micropipette from the cut-off coxa of the first pair of legs and transferred into an ice-cold physiological solution. For the experiments, the hemolymph was diluted two-fold and used within 1 h from accumulation. Ovaries of flies raised from larvae deposited on the same day do not have necessarily the same status of yolk deposition, in other words, they are, to a certain degree, of a different size. Ovaries dissected from 3 to 4-day-old females were used without selection. They were kept on ice in physiological solution till incubation.

2.6. Incubation

One piece of ovary or 5 μl of hemolymph was added to the solution of a radiopeptide **1c**, **1d**, **1e** or **1g** (37 kBq) in 100 μl of physiological saline, homogenized in Eppendorf tube for 30 s using Teflon pestle (Scienceware, Bel-Art Products, Pequannock, USA) and incubated for 1, 30 or 60 min at room temperature. After this time, the homogenate was boiled for 5 min and stored frozen until analyzed. As a stability check, the radiolabeled peptides were treated the same way in the absence of hemolymph or ovaries.

2.7. Enzymatic degradation of tyrosine

Unlabeled tyrosine (0.5 mM) or tritiated tyrosine (37 kBq) were incubated with chosen enzymes L-amino acid oxidase (A), tyrosinase (B) and tyrosine decarboxylase (C) for 1, 30 and 60 min. After this time, the incubation mixture was boiled for 5 min, centrifuged and analyzed by HPLC. The buffers for incubation were 50 mM HEPES pH 7.6 for A and B and 0.2 M acetate buffer pH 5.0 containing 0.5 mM pyridoxalphosphate for C. Degradation products of unlabeled tyrosine were analyzed using Spectra Physics HPLC apparatus, Nucleosil 120 C18 column and gradient of 0.1% TFA and 80% ACN in 0.1% TFA from 0% to 20% for 20 min. Analysis of the tritiated tyrosine degradation products was done by radio-HPLC as described above.

2.8. Identification of tyrosine metabolite

After enzymatic degradation of [^{3}H]Tyr and its radio-HPLC, the unknown radioactive fraction was collected (0.3 ml), lyophilized and the residue again dissolved in redistilled water (0.3 ml); also the evaporated liquid was collected. Both fractions were analyzed by radio-HPLC and activity determined by liquid scintillation spectrometer Beckman 6500.

3. Results and discussion

3.1. Peptide standards

The non-labeled peptides **2a**–**2h** (Table 2) synthesized for the purpose of this study were characterized by different analytical methods (Table 4). Their qualities entitled them to be used as standards for identification of the radiochromatographic fractions (Fig. 1).

3.2. Radio-HPLC analysis

Radio-HPLC of tritiated pentapeptides was performed after 1, 30 and 60 min of incubation alone or with the hemolymph or ovaries. The samples were thawed and centrifuged for 5 min in an Eppendorf centrifuge, and an aliquot of the supernatant was analyzed. Non-labeled standard peptides were detected by UV and their retention times were compared to that of peaks in the radiochromatogram (Fig. 1). The retention times of all radioactive peptides were corrected by the value of the time delay between UV and radiometric detector (0.7 min). The precision of the method was expressed as the coefficient of variation (CV) in %. It was determined by analyzing five replicates of the same biological sample within 1 day. The within-day-precision ranged from 1.5% to 8.7%.

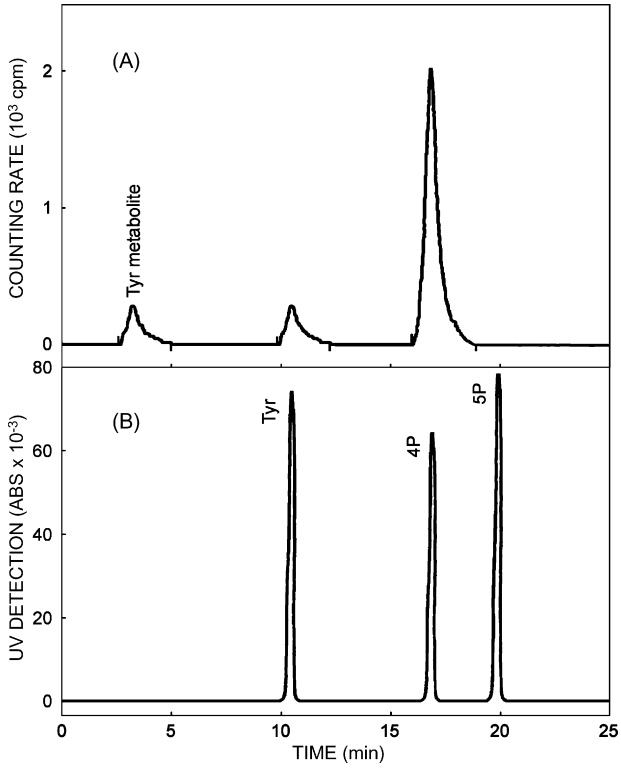

Fig. 1. The HPLC analysis of degradation products of [^{3}H]Tyr^1-5P after 30 min incubation with hemolymph of *Neobellieria bullata*. (A) Radiochromatogram of labeled degradation products and (B) simultaneous UV detection of non-labeled standards added, Tyr, 4P and 5P.

Table 4
Analytical data of peptide standards

Compound	Formula[a]	Amino acid analysis[b]				Electrophoresis[c]		
HPLC elution time (min)[d]	Calc./found (*m/z*)	Tyr	Asp	Pro	Ala	$E^{Gly}_{2.4}$	$E^{His}_{2.4}$	$E^{Pic}_{5.7}$
2a	$C_{17}H_{26}N_4O_7$	–	1.00	1.85	1.03	0.86	0.46	0.49
6.58[e]	398.1/399.2							
2b	$C_{13}H_{23}N_3O_4$	–	–	1.87	1.00	0.45	0.32	–
5.96[e]	284.3/284.3							
2c	$C_8H_{14}N_2O_3$	–	–	0.94	1.00	0.49	0.35	–
8.68[f]	186.2/187.2							
2d	$C_{18}H_{23}N_3O_7$	0.93	1.00	0.94	–	0.88	0.48	0.51
7.33[e]	393.4/394.3							
2e	$C_{13}H_{16}N_2O_6$	0.97	1.00	–	–	0.92	0.52	0.61
8.11[g]	296.1/297.1							
2f	$C_{12}H_{19}N_3O_6$	–	1.00	0.97	1.03	0.91	0.51	0.53
9.54[h]	301.3/302.3							
2g	$C_8H_{14}N_2O_3$	–	–	0.96	1.00	0.48	0.36	–
8.52[f]	186.2/187.2							
2h	$C_9H_{14}N_2O_5$	–	1.00	0.97	–	0.94	0.54	0.63
8.88[h]	230.2/231.0							

[a] Determined with FAB MS technique (VG Analytical, England).
[b] Amino acid analyses were performed on Biochrom 20 instrument (Pharmacia, Sweden).
[c] Electrophoreses were carried out on Whatman 3MM paper (moist chamber, 20 V/cm, 1 h) in 1 M AcOH (pH 2.4) and in a pyridine–acetate buffer (pH 5.7).
[d] For HPLC, a Spectra Physics instrument with a SP 8800 pump, a SP 4290 integrator and Thermo Separation Products Spectra 100 UV detector were used.
[e] Five micrometer LiChrospher WP-300 RP-18 column, 15 cm × 0.4 cm (Merck, Germany), flow rate 1 ml/min, detection at 220 nm, 0–100% gradient of ACN in 0.05 aqueous TFA, 40 min, concentration of peptides in samples analyzed 1 μmol/2.5 ml of 0.05% aqueous TFA.
[f] The same HPLC conditions as described under "e" were used with the exception of the flow rate 0.5 ml/min.
[g] The same HPLC conditions as described under "e" were used with the exception of the flow rate 0.5 ml/min and 60 min gradient.
[h] The same HPLC conditions as described under "e" were used with the exception of the column size 25 cm × 0.4 cm and isocratic mode using 0.05% aqueous TFA as mobile phase.

The recovery of the extraction procedure was evaluated using [^{3}H]Tyr^1-5P calibration solutions at three different concentrations (37, 185 and 370 kBq/ml) by comparison of the extracted and the applied radioactivity. The resulting value of recovery was between 82.3% and 94.5% ($n = 4$) with precision range from 2.6 to 3.7 (CV%). The linearity of the radiometric detector response was verified using [^{3}H]Tyr^1-5P calibration solutions in the range of 200–10,000 Bq with an average correlation coefficient of 0.997 ($n = 4$). The absolute detection limit in the system, defined by a signal-to-noise ratio of 3, was assigned for the 5P in the range of 80–150 Bq, which corresponds to 56–106 fmol for 1.42 TBq/mmol (Table 3). An illustrative radiochromatogram is given in Fig. 1A. It shows an analysis of the reaction mixture of [^{3}H]Tyr^1–5P after 30 min incubation with hemolymph. Fig. 1B shows the UV detection of non-radioactive standards added.

3.3. Degradation products of 5P

The results obtained by radio-HPLC of 5P tritiated in different positions of the peptide chain after incubation with hemolymph are summarized in Fig. 2. They proved relatively quick degradation having 4P as the main degradation product. Surprisingly, among the shorter peptide sequences, no tripeptide H-Tyr-Asp-Pro-OH (3P) was detected. Because of the problems of separation of 3P and 4P under the basic gradient HPLC conditions used, an isocratic elution using 4% ACN was found suitable to separate a mixture of 3P and 4P standards. However, even under these conditions, the tripeptide was not detected in the degradation mixture of 5P. This fact was verified by measuring the time delay between UV and radiometric detector.

3.4. Tyrosine degradation by enzymes

In the experiments with 5P containing [^{3}H]Tyr (**1g**), tritiated tyrosine was detected in the incubation mixture together with an unknown metabolite at the beginning of radio-HPLC

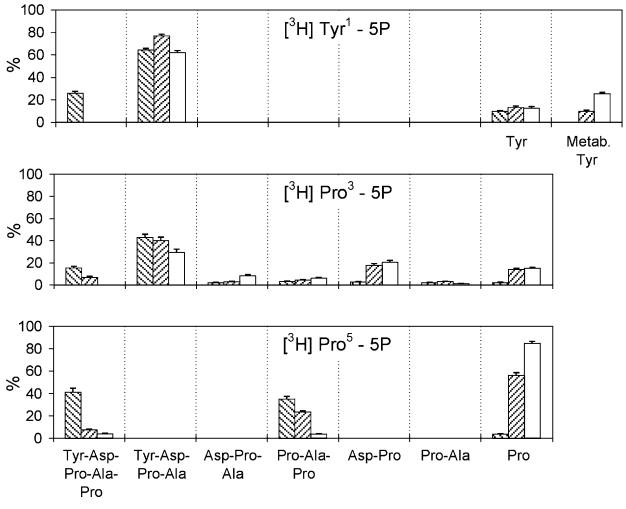

Fig. 2. The time course of metabolite formation of 5P labeled in different positions of the peptide chain during incubation with hemolymph of *Neobellieria bullata* analyzed by radio-HPLC. Time of incubation: 1 ▨, 30 ▨, or 60 □ min.

Table 5
Differences in the degradation of in position 1 tritiated 5P and 4P

	Incubation (min)	[^{3}H]Tyr^1-5P (%)	[^{3}H]Tyr^1-4P[a] (%)
Hemolymph	1	26	85
	30	0	71
	60	0	54
Ovaries	1	78	84
	30	37	10
	60	25	4

[a] Data taken from experiments in paper [3].

analysis (Fig. 1A). To elucidate the structure of the metabolite, we treated non-labeled tyrosine and tritiated tyrosine with three different model enzymes, i.e. L-amino acid oxidase, tyrosine decarboxylase and tyrosinase. When using unlabeled tyrosine, none of the enzymes used yielded a product in the same position as the unknown metabolite. On the other hand, the incubation of [^{3}H]Tyr with tyrosinase yielded this metabolite immediately. Taking into account the fact that tyrosinase oxidizes tyrosine in positions having tritium and in positions destabilizing bound tritium, we hypothesized that the unknown metabolite is water. This assumption was proved by the same retention time of the metabolite and radioactive water collected during lyophilisation of the metabolite solution. The activity of this water sample formed $93 \pm 3\%$ of the ^{3}H degraded of [^{3}H]Tyr, while the dissolved lyophilisate, having a different retention time, contained only $2.3 \pm 0.2\%$ of the tyrosine radioactivity.

3.5. Comparison of 5P degradation in hemolymph and ovaries

The total amount of the residual non-degraded radiolabeled 5P after different times of incubation was used to express 5P degradation. From the obtained values (Table 5), it is evident that the degradation in hemolymph is much quicker than that in ovaries. This is in contrast to the degradation of 4P [3], which was degraded quicker in the ovaries. This would suggest that different enzymatic systems degrade the peptides in hemolymph and in the ovaries. Meanwhile, there is quick cleavage of the N-terminal tyrosine from the 4P in ovaries, whereas the C-terminal proline is hindering this cleavage in the case of 5P. On the other hand, the 4P is more stable in hemolymph than in ovaries; however the cleavage of the C-terminal proline from 5P is very quick.

4. Conclusion

The experiments showed the convenience of radio-HPLC for monitoring of the degradation of peptides in biological fluids. Radiolabeling in different positions of the peptide chain allows to determine the decisive step of the degradation; in our case, it is the splitting off the C-terminal proline from the pentapeptide 5P. In comparison to the LC/MS analysis of metabolites used in our previous paper [3], the radio-HPLC gives more precise and quite unambiguous results. The results also support our previous finding of the high biological activity of both the 5P and 4P [3]. Even if 5P is degraded quite quickly (proline is split off the pentapeptide), the biologically active tetrapeptide is formed, that is degraded much slower.

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