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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-