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Note

Ion-pair high-performance liquid chromatography of the insect neuropeptide proctolin and some analogs

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Although the potent insect myotropic peptide proctolin, Arg-Tyr-Leu-Pro-Thr, has been characterized by a number of methods including paper and thin-layer chromatography, high-voltage paper electrophoresis and pharmacological assay[1,2], a rapid, unambiguous means of identification and purity assessment has not been available. Recently, Holman and Cook[3,4] have reported the analysis of proctolin in insect extracts by high-performance liquid chromatography (HPLC) of the phenylthiocarbamyl derivative. In this paper we report on the use of ion-pair reversed-phase HPLC[5,6] for the analysis and characterization of underivatized proctolin and its separation from some closely related analogs.

EXPERIMENTAL

Reversed-phase HPLC was carried out on a 30 cm × 3.9 mm I.D. μBondapak fatty acid analysis column (Waters Assoc., Milford, MA, U.S.A.) using a Waters liquid chromatographic system equipped with a Model 6000A pump, a Model U6K sample injector and a Schoeffel Model SF-770 variable-wavelength detector operated at 225 nm. All chromatography was performed at room temperature. Distilled water and HPLC grade acetonitrile (Caledon Labs., Georgetown, Canada) were used for the mobile phases. Ion-pairing reagents used were PIC Reagent B-6 obtained from Waters Assoc., trifluoroacetic acid (TFA) from Eastman-Kodak (Rochester, NY, U.S.A.), heptafluorobutyric acid (HFBA) from Pfaltz and Bauer (Stamford, CT, U.S.A.) and orthophosphoric acid and trichloroacetic acid (TCA) from Fisher Scientific (Toronto, Canada). Before degassing and use, the acetonitrile was filtered through a Millipore FHUP 0.50-μm filter and the water containing the ion-pairing reagent was filtered through a Millipore HAWP 0.45-μm filter. The column was equilibrated to new solvents for at least 30 min.

The pentapeptides, synthesized and purified in this laboratory by standard procedures[7,8], were dissolved in distilled water (1 μg/μl) and 1–10 μl were injected.

RESULTS AND DISCUSSION

Proctolin was separated from analogs differing by only a single amino acid by ion-pair reversed-phase HPLC on a μBondapak fatty acid analysis column using

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acetonitrile–water mixtures (Table I). Marked alterations in retention times were achieved by varying the ion-pairing reagent. In agreement with previous studies of small peptides[9] the order of elution paralleled the hydrophobicity of the constituent L-amino acids. Similar results were obtained when a μBondapak C_{18} column was used.

TABLE I

EFFECT OF ION-PAIRING REAGENTS ON THE RETENTION TIMES OF PROCTOLIN AND SOME RELATED PENTAPEPTIDES ON A μBONDAPAK FATTY ACID ANALYSIS COLUMN

Peptide	*Retention time (min)*						
	*H_3PO_4**	*TFA**	*TFA***	*TCA***	*HFBA***	*PIC Reagent B-6***	*Sodium hexane sulfonate***
Arg-Tyr-Leu-Pro-Thr	7.1	11.3	3.5	5.5	8.2	5.1	3.7
Arg-Tyr-Leu-Pro-Ala	7.2	12.6	3.7	6.1	9.4	5.7	3.9
Arg-Tyr-Leu-Ala-Thr	4.6	7.0	3.0	4.1	6.9	4.4	3.4
Arg-Tyr-Ala-Pro-Thr	3.1	4.3	2.4	3.3	4.5	3.4	2.7
Arg-Ala-Leu-Pro-Thr		5.2	2.6	3.6	5.2		
Ala-Tyr-Leu-Pro-Thr	12.5	16.4	4.0	5.2	5.4	4.4	2.4
Arg-Tyr-Leu-Pro-D-Thr	10.2	17.4	3.8	6.7	10.1	5.8	4.6
Arg-Tyr-Leu-D-Pro-Thr	9.7	16.1	4.1	7.2	11.2	5.7	4.6
Arg-Tyr-D-Leu-Pro-Thr	16.5		5.5	10.1	14.8	8.0	7.4
Arg-D-Tyr-Leu-Pro-Thr	21.7		5.7	10.5	15.9	8.7	5.7
D-Arg-Tyr-Leu-Pro-Thr	14.3	24.8	4.5	8.4	12.7	7.2	4.7
Arg-Tyr-Leu-Pro-Ser	5.9	9.3	3.0	4.8	7.2	4.5	3.4
Arg-Phe-Leu-Pro-Thr	15.0		5.3	10.1	16.1	8.5	6.0

* 5 m*M* in acetonitrile–water (1:9); flow-rate 1.5 ml/min.
** 5 m*M* in acetonitrile–water (1:4); flow-rate 1.5 ml/min.

Proctolin and the five possible diastereoisomers resulting from the replacement of each of the amino acids in turn with the respective D-amino acid could also easily be separated. The diastereoisomers all eluted more slowly than proctolin with highest hydrophobicity being found for [D-Tyr^2]-proctolin. Separation of peptide diastereoisomers has previously been discussed in terms of conformational differences between the isomers[10–12].

All the peptides eluted more slowly with PIC Reagent B-6 (5 m*M* hexane sulfonic acid containing sufficient acetic acid to yield a pH of about 3.5) than with 5 m*M* sodium hexane sulfonate (pH 6.4) due to suppression of ionization of the carboxyl group and more effective ion-pairing with the α-amino group at the lower pH.

HFBA, which to our knowledge has not been reported previously as an ion-pairing reagent, gave excellent resolution of proctolin and most of its analogs. Fig. 1 illustrates results with this reagent. Slight increases in the capacity factors and small changes in the separation factors for the peptides were observed when the amount of HFBA was increased from 5 m*M* to 25 m*M*. Like TFA[13], HFBA may be readily removed by lyophilization so systems containing this reagent should be useful for preparative chromatography of peptides.

Our results further illustrate the utility of ion-pair reversed-phase HPLC using isocratic systems in separating and characterizing small, underivatized, closely related

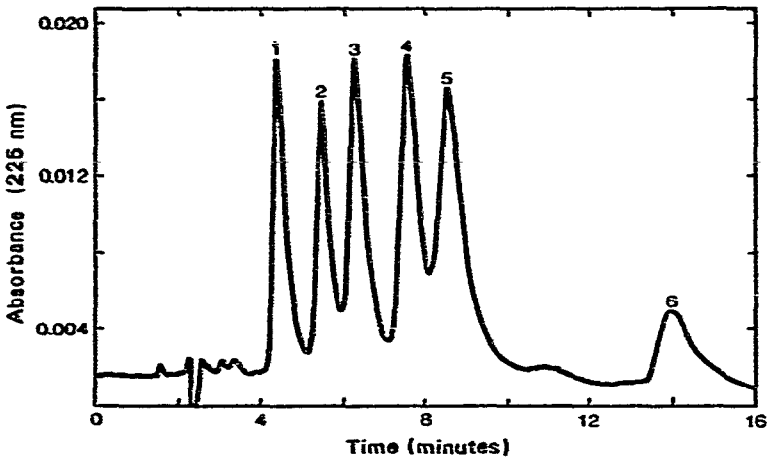

Fig. 1. Elution pattern of proctolin and five analogs on a μBondapak fatty acid analysis column with acetonitrile–water (1:4) containing 5 m*M* HFBA as eluent at a flow-rate of 1.5 ml/min. Peaks: 1 = [Ala^3]-proctolin; 2 = [Ala^1]-proctolin; 3 = [Ala^4]-proctolin; 4 = proctolin; 5 = [Ala^5]-proctolin; 6 = [Phe^2]-proctolin.

peptides. More specifically they indicate that this technique will be very convenient for determining the homogeneity of synthetic proctolin and its analogs, including the detection of diastereoisomeric impurities, as well as for monitoring the stability of solutions of these peptides. It is also anticipated that ion-pair reversed-phase HPLC can be employed to simplify the lengthy isolation procedure for proctolin[14] and, by separating proctolin from other insect myogenic peptides, will permit the bioassay of low levels of proctolin in relatively crude extracts prepared from either whole insects or specific tissues.

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