Journal of Chromatography, 346 (1985) 390–395
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 001

Note

Reversed-phase high-performance liquid chromatography of natural and synthetic sauvagines

LUISA RUSCONI* and PIER CARLO MONTECUCCHI
Farmitalia Carlo Erba, Chemical Research & Development, Via dei Gracchi 35, 20146 Milan (Italy)
(Received July 5th, 1985)

Sauvagine (SAU) is a peptide isolated from the skin of the South American frog *Phyllomedusa sauvagei*[1,2], and occurs also in skin extracts from other *Phyllomedusa* species[3]. It consists of a straight chain of 40 amino acid residues with distinct hydrophobic characteristics, the sequence of which is shown in Fig. 1. In addition to displaying activity towards the cardiovascular system and the gastrointestinal tract, SAU stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary gland[5] both *in vivo* and *in vitro*. The peptide shows considerable similarities in biological activities with the corticotropin-releasing factor (CRF) isolated from the sheep hypothalamus[6] and other homologous hypophysiotropic factors isolated from rat[7], bovine[8] and caprine[9] hypothalami, and also with urotensin I (U_I), detected in the urophysis of two different species of bony fishes[10,11]. These peptides are also closely related in structure, showing a sequence identity of at least 50% (see Fig. 1).

Natural SAU is known to occur in two forms with different electrophoretic mobilities, SAU I and SAU II, but displaying the same biological spectrum. The presence of one additional carboxyl group in SAU II (identified as [Glu^{23}]SAU I) was demonstrated by an automatic version of the Edman degradation and confirmed by the pseudo-titration curves of the two forms of sauvagine in isoelectric focusing–electrophoresis[12]. Moreover, similarly to homologous ovine CRF[6] and to other methionine-containing peptides, *e.g.*, substance P[13], and tryptophyllin-13[14], SAU is likely to be obtained partially in the methionine sulphoxide form by virtue of the purification procedure. [MetO^{17}]SAU was found not to be significantly different from SAU in biological potency[12].

The conventional synthesis of the (18–40) fragment[15], as well as the solid-

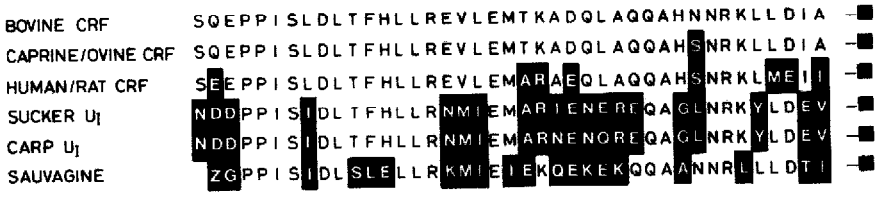

Fig. 1. Primary structures of native peptides with corticotropin-releasing activity. The one-letter system of abbreviations for the amino acids has been used[4]. In particular, Z denotes pyrrolidonecarboxylic acid and the symbol ■ represents the amino group blocking the carboxyl end of the amino acid chain.

0021-9673/85/$03.30

phase syntheses of the (17–40) fragment[16] and of the complete amino acid sequence[17], have recently been described. The product corresponding to the tetracontapeptide is now commercially available from different chemical companies. Nevertheless, no chromatographic comparison between the natural compound and the synthetic ones, and no complete characterization of extractive SAU by high-performance liquid chromatography (HPLC), has been reported. In this paper we describe the use of reversed-phase high-performance liquid chromatography (RP-HPLC) for the identification and resolution of the different forms of natural SAU, and for the analysis of two synthetic SAU samples.

EXPERIMENTAL

RP-HPLC was performed on a Hewlett-Packard 1084B apparatus, equipped with an HP 1040 diode-array detector controlled through an HP 85 computer.

One synthetic sample of SAU (referred to as sample 1) was obtained from Sigma (St. Louis, MO, U.S.A.): the other one (referred to as sample 2) was a kind gift from Dr. W. Vale (Salk Institute, La Jolla, CA, U.S.A.).

The natural compound was isolated as previously reported from the methanol extracts of skin from *Phyllomedusa sauvagei*[1,2]. A semipurified pool (referred to as pool 1) was collected after gel filtration on Sephadex G-50 superfine (Pharmacia, Uppsala, Sweden). A part was employed for the HPLC characterization, whereas another amount was further purified on a DEAE-Sephacel (Pharmacia) column. Two other pools were collected, containing SAU I (pool 2) and SAU II (pool 3), respectively, and they were also analyzed by HPLC. All reagents were of analytical grade.

Quantitative amino acid analyses were carried out using a Kontron Chromakon 500 amino acid analyzer. Samples for amino acid analysis were hydrolyzed at 110°C for 48 h in 6 *M* hydrochloric acid under nitrogen atmosphere. The samples for chromatography were freshly dissolved in water (*ca.* 1 mg/ml) and analyzed on a μ-Bondapak C_{18} column (300 × 3.9 mm I.D.; Waters Assoc., Milford, MA, U.S.A.) using the following eluent systems: (I) A = 0.05% trifluoroacetic acid, B = acetonitrile; (II) A = 0.01 *M* sodium acetate, B = acetonitrile; (III) A = 0.02 *M* ammonium acetate, B = acetonitrile, always with gradient elution from 30 to 55% B in 25 min. All separations were performed at room temperature (*ca.* 25°C) and at a flow-rate of 1 ml/min. The detection wavelength was fixed at 220 nm.

RESULTS AND DISCUSSION

Pool 1 gave the elution pattern shown in Fig. 2 when analyzed on a μBondapak C_{18} column with eluent system I. Two major, well resolved peaks (A and C) were present in the chromatogram, each being accompanied by a smaller peak at slightly higher retention time (peaks B and D). The retention time of peak C was coincident with that of synthetic peptides 1 and 2 (see Table I). This coincidence was confirmed when mixtures of an aliquot from pool 1 with samples 1 and 2 were analyzed under the same conditions; therefore peak C was attributed to native SAU I.

Peak A in Fig. 2 accounted for a product occurring in approximately equal amount to SAU I in pool 1. It could be identified as [MetO^{17}]SAU I by comparison with a synthetic standard, obtained through mild oxidation (0.18% hydrogen per-

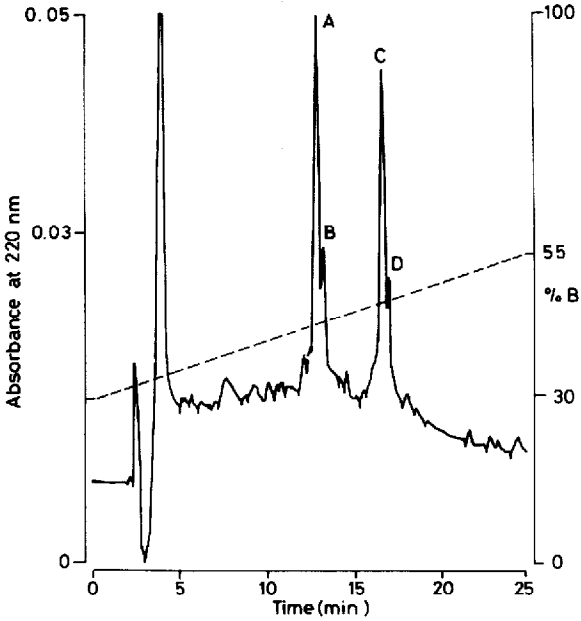

Fig. 2. RP-HPLC profile of extractive SAU from pool 1. Conditions: column, μBondapak C_{18}; eluent A, 0.05% trifluoroacetic acid, eluent B, acetonitrile; gradient, as shown by the dotted line; flow-rate, 1 ml/min; sample load, 60 μg. For peaks A–D, see Table I.

oxide in 0.05 *M* acetic acid, 15 min at room temperature, peptide concentration *ca.* 0.5 mg/ml) of sample 1. The elution profile of pool 1 coinjected with synthetic [MetO[17]]SAU I is shown in Fig. 3a. A comparable amount of native SAU was also oxidized by the above method: from the elution pattern in Fig. 3b, it is seen that after this treatment only two products were detected, with the same retention times as peaks A and B in Fig. 2. The first peak could definitely be attributed to [MetO[17]]SAU I, while the second one could only correspond to an oxidized de-

TABLE I

RP-HPLC BEHAVIOUR OF TWO SYNTHETIC SAU SAMPLES, OF NATURAL SAU I AND II AND THEIR RESPECTIVE METHIONINE SULPHOXIDE FORMS

Systems: (I) A = 0.05% trifluoroacetic acid; B = acetonitrile; (II) A = 0.01 *M* sodium acetate; B = acetonitrile; (III) A = 0.02 *M* ammonium acetate; B = acetonitrile. Samples were always eluted with a gradient from 30 to 55% B in 25 min.

Peak	*Product*	*Retention time (min)*		
		System I	*System II*	*System III*
A	[MetO[17]]SAU I	12.7	10.9	12.1
B	[MetO[17]]SAU II	13.1	8.8	10.1
C	SAU I	16.4	14.5	15.3
	Sample 1	16.4	14.5	15.3
	Sample 2	16.4	14.5	15.3
D	SAU II	16.7	12.5	13.5

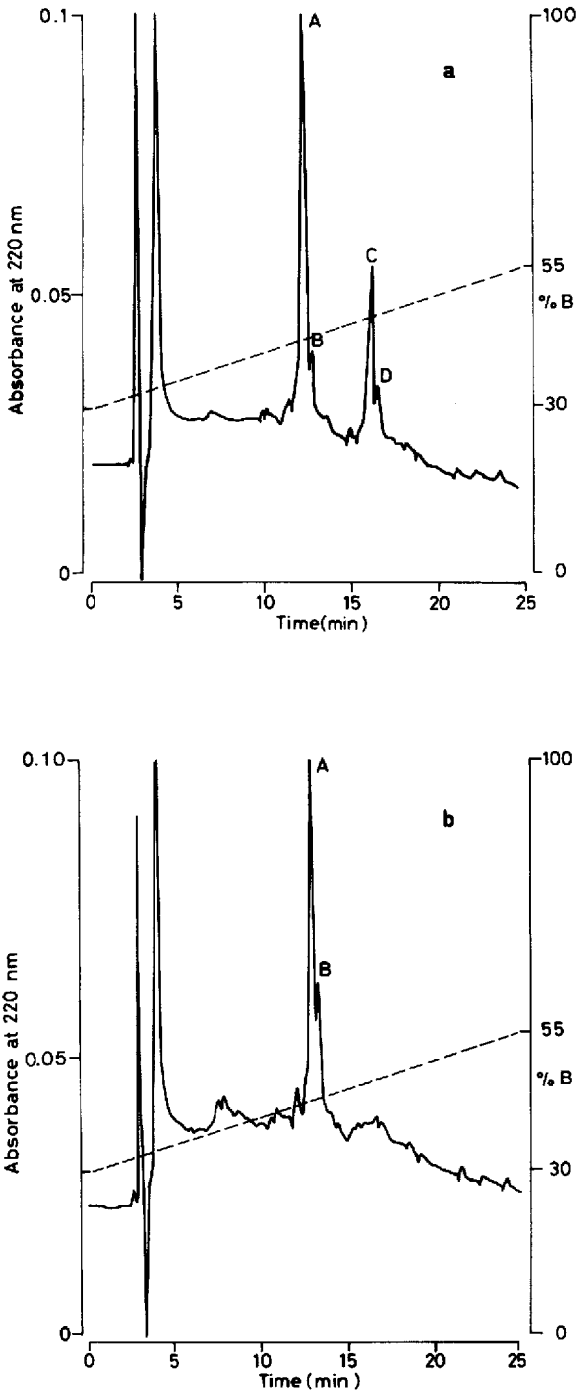

Fig. 3. RP-HPLC profiles of synthetic [MetO[17]]SAU I (15 μg) and extractive SAU from pool 1 (60 μg) (a) and of pool 1 (30 μg) after oxidation with peracetic acid (b). Conditions as in Fig. 2.

rivative of the product giving peak D in Fig. 2. As a consequence, peaks B and D were supposed to be [MetO[17]]SAU II and SAU II, respectively. In order to confirm this, pool 1 was further purified on a DEAE-Sephacel column under suitable conditions in order to separate SAU I and SAU II[1]. Moreover, since the amino acid analysis of peak C in Fig. 2 did not fully correspond to that expected for SAU I, a further purification step was also necessary to get satisfactorily pure peptides. The two SAU-containing pools from DEAE-Sephacel chromatography were analyzed by the same method as for pool 1, both of them yielding two peaks with the expected retention times. Therefore, the products detected as peaks B and D in Fig. 2 could be ascribed to native [MetO[17]]SAU II and SAU II, respectively.

The chromatographic system so far employed (system I) was found to provide very good resolution between SAU I, II and their respective methionine sulphoxide forms. The method also allowed quick and substantial recovery of the peptides after lyophilization (more than 80%, based on reinjection).

The retention times observed with this method were quite high for SAU I and II, in agreement with their high content of hydrophobic amino acids. The relatively apolar character of the two peptides is further increased by the low pH of the buffer: both of them are fully protonated, that is, quite hydrophobic, due to their acidic isoelectric points. On the contrary, the oxidized forms were less strongly retained, because of the presence of the very polar sulphoxide function in the molecules.

To improve the resolution between SAU I and II, the system was modified by the introduction of 0.01 *M* sodium acetate as aqueous buffer (system II). Gradient elution with acetonitrile as organic modifier was performed on pool 1, and the results

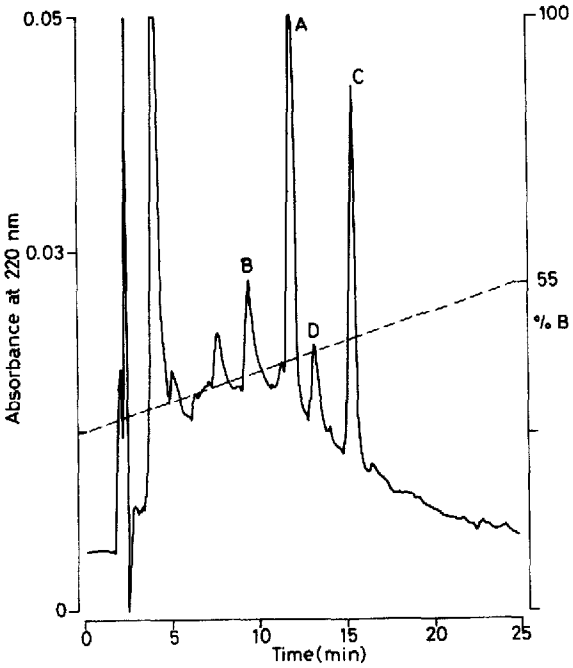

Fig. 4. RP-HPLC profile of extractive SAU from pool 1. Eluent A, 0.01 *M* sodium acetate. Other conditions as in Fig. 2.

are shown in Fig. 4. Four peaks were present in the chromatogram, and readily identified by comparison with the synthetic standards and the pure native peptides obtained in advance (see above). [MetO^{17}]SAU II was eluted first, followed by [MetO^{17}]SAU I, then the two non-oxidized peptides in the same order, that is, reversed with respect to that obtained in first chromatographic system. This reversal is due to the pH of the mobile phase (about 6.6, *cf.*, about 2.4 for the buffer used in system I), which causes dissociation of the carboxyl functions: as a consequence, SAU II and [MetO^{17}]SAU II, which contain one additional ionized group, are eluted more rapidly than their respective [Gln^{23}] analogues. The method employing system II provides full resolution of the four SAU forms. A satisfactory separation was still observed (see Table I) when sodium acetate in the buffer was replaced by the corresponding ammonium salt (system III), to allow for recovery of the products after lyophilization. Moreover, the peptides obtained by preparative HPLC with pool 1 according to this method were already sufficiently pure, thus eliminating the need for the last purification step by ion-exchange chromatography.

CONCLUSION

We have reported the characterization of the two sauvagine forms by use of reversed-phase high-performance liquid chromatography. Their retention times were compared with those of two synthetic samples under different experimental conditions. The demonstration of the identity of natural SAU I and synthetic SAU, here reported for the first time, is particularly helpful for a more complete pharmacological investigation of the rôle of this molecule in mammalian tissues.

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