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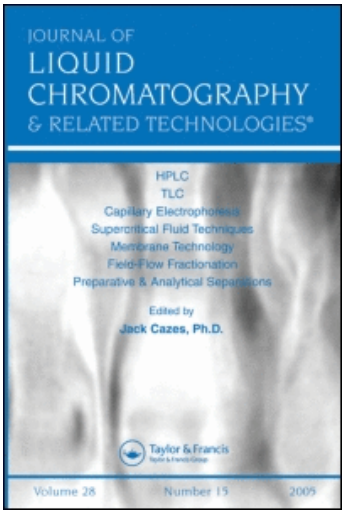

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HPLC PURIFICATION OF SOLID-PHASE GENERATED SYNTHETIC BOMBESIN

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ABSTRACT

A scheme based on ion-exchange and reverse-phase high pressure liquid chromatography has been utilized for the semi-preparative and preparative purification of the solid-phase generated model peptide bombesin. The final product showed a purity ≥ 99% in analytical reverse-phase high pressure liquid chromatography and was identical to authentic bombesin as demonstrated by different physico-chemical and biological criteria. The results are discussed and compared to those obtained using countercurrent chromatography.

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INTRODUCTION*

Bombesin (BN) and related peptides are involved in a variety of peripheral and central neural functions (1-4). Moreover, BN can act as an autocrine growth factor in malignant and normal cells (5-8).

In view of the wide spectrum of biological activities, many research reports regarding synthesis, purification and structural characterization of these molecules have appeared in the recent literature (9-14). The synthesis of BN and re-

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Standard abbreviations for amino acids and peptides are according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature in **European. J. Biochem.** 138, 9-37 (1984). Other abbreviations include: BHA, benzhydrylamine (resin); Boc, tert-butyloxycarbonyl; CCC, countercurrent chromatography; DCC, N,N-dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA, N,N-diisopropylethylamine; DMF, N,N-dimethylformamide; EMS, ethyl methyl sulphide; Et_2O, ethyl ether; EtOAc, ethyl acetate; EtOH, ethanol; HOAc, acetic acid; HOBt, 1-hydroxybenzotriazole; HPLC, high-pressure liquid chromatography; IPA, isopropylalcohol; MeOH, methanol; NMM, N-methylmorfoline; RP, reverse phase; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin-layer chromatography; Tos, 4-toluenesulfonyl.

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lated peptides, due to the presence of residues like Met, Trp, His, Arg, Gln and Asn, is often hampered by a variety of side-reactions which results in complex mixtures that must be purified in order to obtain peptides suitable for structural and biological investigations. A method for the rapid semi-preparative purification of solid-phase generated crude BN, based on ion-exchange and reverse-phase HPLC, is presented in this paper. The purification scheme, applicable also to related peptides, can be readily expanded to large scale preparation of these important molecules.

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MATERIALS AND METHODS

All amino acids, except glycine, were of L-configuration. Protected amino acids for the synthesis were obtained from Bachem or Peninsula Laboratories and were checked by TLC and amino acid analysis prior to use. BHA hydrochloride resin, 2% divinylbenzene, 100-200 mesh, with a capacity of 0.5 mmol/g was also from Peninsula.

Peptide Synthesis.

Benzhydrylamine hydrochloride resin (3 g) was washed with 3 x 25 mL each of DCM, DMF, DCM and neutralized by a 0.5 min prewash followed by a 2 min treatment with NMM in DCM. The resin was thoroughly washed with DCM, IPA, DCM and reacted during 2 h with Boc-Met-OH (1.12 g, 4.5 mmol) dissolved in 10 mL DCM and 1 M DCC in DCM (4.5 mL). The resin was then washed with 3 x 25 mL DCM, DMF, DCM, neutralized

with 10% NMM, as described above, and the residual free amino groups acylated with excess N-acetylimidazole (15) (1.65 g, 15 mmol) in 10 mL DCM for 1 h. A 5 mg sample, submitted to quantitative ninhydrin reaction (16), gave less than 0.06 μmol/g free amino groups. An other sample, after hydrolysis with 12 N HCl-propionic acid, gave an incorporation of 0.115 mmol Met/g resin. This resin was used for the manual step-wise assembly of the BN sequence. Single coupling in DMF with 3-fold excess preformed symmetric anhydrides (90-120 min) was routinely employed at each residue. The side-chain functionalities of Arg and His residues were protected by the Tos group. Asn and Gln residues were coupled for 120 min using the corresponding preformed HOBt esters (5-fold excess) in DMF. 50% TFA in DCM containing 0.1% indole was used for removal of Boc groups throughout the synthesis. A 5% DIEA solution in DCM was used for neutralization. After completion of the synthesis, the peptide-resin was stirred with 30 mL anhydrous liquid HF at 0° for 1 h in the presence of 3 mL anisole and 0.6 mL EMS. Volatile materials were removed "in vacuo" and the residue washed with 4 x 25 mL Et_2O and EtOAc and finally extracted with 3 x 25 mL 100% HOAc. The extracts pooled and lyophilized gave the crude product which was utilized for the separation study.

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HPLC

1) Preliminary analytical characterizations were made on a Waters Associates system equipped with a M660 programmer, 2

M6000 pumps and a M450 detector on Waters μ-Bondapak C-18 columns (3.9 mm i.d. x 300 mm). Elution was accomplished by a binary solvent system. Solvent A consisted of 0.1% TFA in water, solvent B contained 0.1% TFA in 75% acetonitrile.

2) A Waters M650 system equipped with M990 and M484 detectors and a M745 integrator was used for ion-exchange chromatography with a Protein-Pak SP 5PW (7.5 mm i.d. x 75 mm) column which was eluted with a linear gradient of sodium acetate and sodium chloride. Eluant A) was 0.02 M sodium acetate pH = 5.0; eluant B) 0.02 M sodium acetate, 0.5 M sodium chloride pH = 5.0. Gradient conditions were as follows: linear from 100% a) to 100% B) in 30 min; 100% B) for 10 min, then 20 min riequilibration with 100% A). Flow-rate was 1.0 mL/min. The sample was dissolved in eluant A and filtered on Millex-HV13 0.45 μm.

3) A Waters M600 system equipped with a M990 detector was used for analytical RP-chromatography with a 5 μ Delta-Pak C-18 100 Å (3.9 mm i.d. x 150 mm) column which was eluted with a linear gradient. Eluant A) was 0.1% TFA in water; eluant B) 0.1% TFA in acetonitrile. Gradient conditions were as follows: linear from 20% B) to 40% B) in 20 min at a 1.0 mL/min flow-rate, then to 90% B) in 1 min increasing the flow-rate to 1.5 mL/min, and then for 5 additional min. Riequilibration with 20% B) at 1.5 mL/min flow-rate.

4) A Waters M600 system equipped with a M484 detector and a M745 integrator was used for semi-preparative RP-chromatography with a 15 μ Delta-Pak C-18 100 Å (7.8 mm i.d. x 300 mm) column which was eluted with a linear

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gradient. Eluant A) was 0.1% TFA in water; eluant B) 0.1% TFA in acetonitrile. Gradient conditions were as follows: linear from 15% B) to 40% B) in 60 min at a 4 mL/min flow-rate.

4) Large scale separations were performed, in the 20-200 mg range, on a Kronwald Sepachrom PPC C-18, 100 Å, HD-SIL, 20-45 μ (26 mm i.d. x 313 mm) glass column. Up to 1 g of crude material was separated on (26 mm i.d. x 500 mm) glass column. Both columns were eluted with a linear gradient. Eluant A) was 0.1% TFA in water; eluant B) 0.1% TFA in acetonitrile. Gradient conditions were as follows: linear from 5% B) to 70% B) in 60 min at a 10-20 mL/min flow-rate.

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Amino acid analysis

Amino acids analyses were performed on a Carlo Erba Mod. 3A 28 automatic analyzer. Peptides were hydrolyzed with 6 N HCl at 110° C for 22 h, in evacuated sealed tubes. Peptide-resins were hydrolyzed with a mixture of 12 N HCl-propionic acid (1:1, v:v), in evacuated sealed tubes for 2 h at 130° C (17).

Biological assay

Dispersed acini from guinea pig pancreas, prepared according to (18) were suspended in 150 mL of standard incubation solution and samples (250 μL) were incubated for 30 min at 37°C. Amylase activity was determined by the method of Ceska et al. (19, 20) using the Phadebas reagent. Amylase release was calculated as the percentage of amylase activity

in the acini at the beginning of the incubation that was released into the extracellular medium during incubation.

Fast atomic bombardment mass spectra

Positive ion FAB mass spectra were recorded on a VG 70-250 (Manchester, UK) instrument using an M-SCAN FAB gun operating at 8 KV and 1 mA. Xenon was used as bombing gas. Peptide samples were dissolved in water-ethanol and 1-2 µL loaded onto the probe tip previously coated with a glycerol-thioglycerol mixture. The spectra were recorded at a 5KV accelerating voltage and ion mass assignment was achieved by peak matching to cesium iodide clusters (21).

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Thin-layer Chromatography

TLC of the peptides was run on HP-KF silica gel plates (Whatman) using as eluant the upper phase of the mixture: Butanol-1/Pyridine/0.1% AcOH; 50/30/110; v/v/v.

RESULTS

BN has been synthesized by solid-phase techniques on BHA resin.It should be noted that, since the goal of the present research was to perform suitable conditions for efficient and rapid purification of heterogeneous synthetic material, no particular precautions like: type of resin,

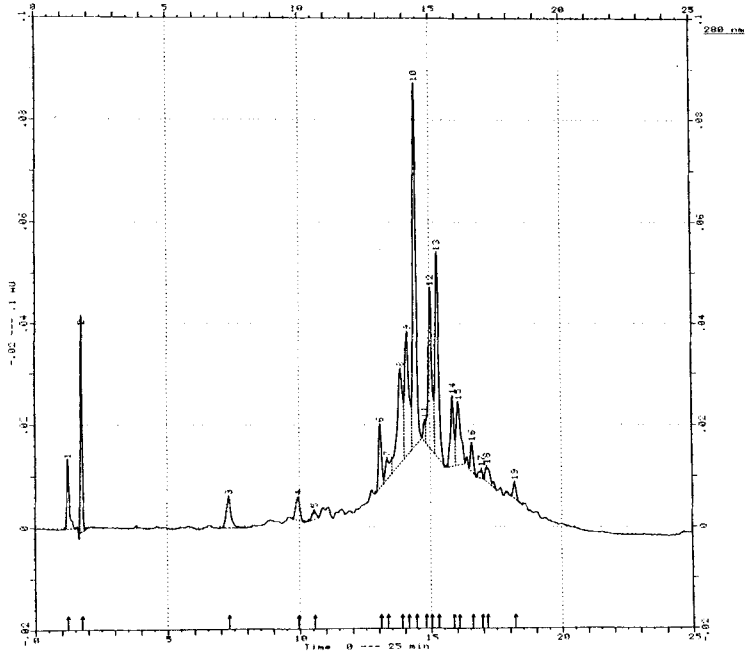

Fig. 1

a) RP-HPLC of crude synthetic BN on a 5μ Delta-Pak C-18, 100 Å, (3.9 mm i.d. x 300 mm) column. Buffers: 0.1% TFA in water = A; 0.1% TFA in acetonitrile = B. Gradient: linear from 20% to 40% B in 20 min. Flow-rate: 1 mL/min. Load: 25 μL, ≈ 50 μg. Sampling time: 28 msec. Y-Scale: 0.12 AU/FS. Wavelenght: 280 nm. Resolution: 2nm. Time range: 0-25 min. Interval: 1 sec. Paper speed: 10 mm/min. The major peak with a retention time of 14.14 min corresponds to BN as it was demonstrated by enrichment experiments with authentic BN (data not shown).

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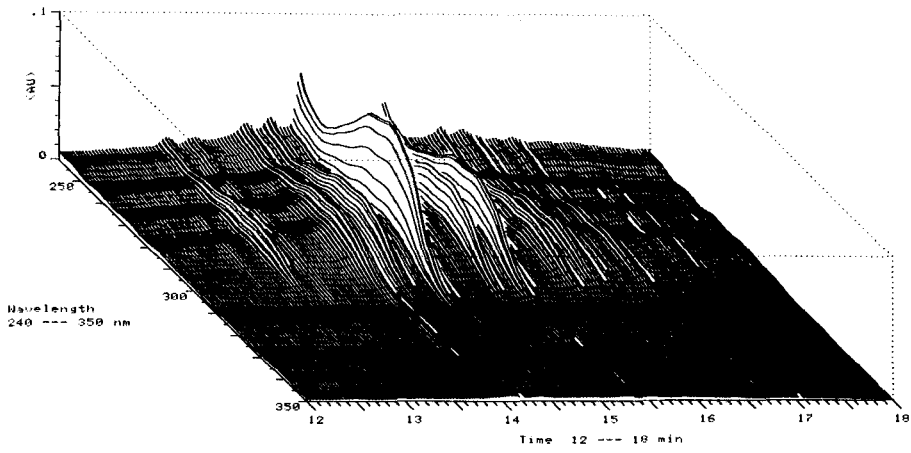

Fig. 1b

b) 3D-Plot of the spectrum analysis utilizing a photodiodes array M990 detector of the separation reported in Fig. 1a. Sampling time: 28 msec. Y-Scale: 0.1 AU/FS. Wavelenght: 240-350 nm. Resolution: 2nm. Time range: 12-18 min. Interval: 1 sec. Paper speed 30 mm/min

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quantitative monitoring, double coupling, capping, etc., were taken to optimize the synthetic protocol used. Treatment of the peptide-resin with HF in the presence of scavengers gave the crude material which was utilized to set up a purification scheme based on HPLC methods.

Fig. 1a shows the profile obtained injecting the crude material, directly after HF cleavage, on a 5μ Delta-Pak C-18, 100 Å, (3.9 mm i.d. x 150 mm) column which was eluted with a linear gradient of water/acetonitrile, as described

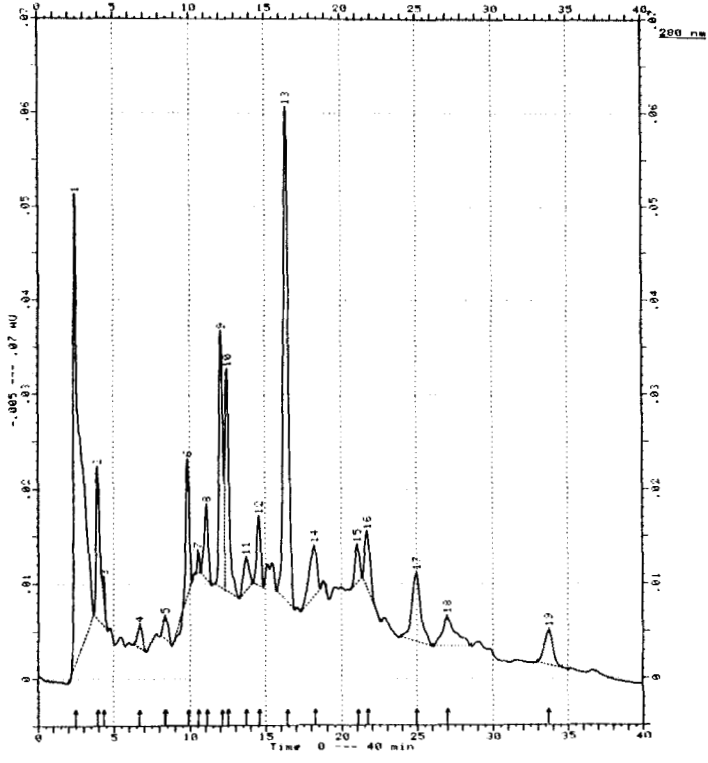

Fig. 2

a) Ion-exchange HPLC of crude synthetic BN on a Protein-Pak SP 5PW (7.5 mm i.d. x 75mm) column. Buffers: 0.02 M sodium acetate, pH 5.0 = A; 0.02 M sodium acetate, 0.5 M sodium chloride, pH 5.0 = B. Gradient: linear from 100% A to 100% B in 30 min. Flow-rate: 1 mL/min. Load: 100 µL, ≈ 200 µg. Sampling time: 26 msec. Y-Scale: 0.075 AU/FS. Wavelenght: 280 nm. Resolution: 2nm. Time range: 0-40 min. Interval: 1 sec. Paper speed: 5 mm/min. The major peak with a retention time of 16.42 min corresponds to BN as it was demonstrated by enrichment experiments with authentic BN (data not shown).

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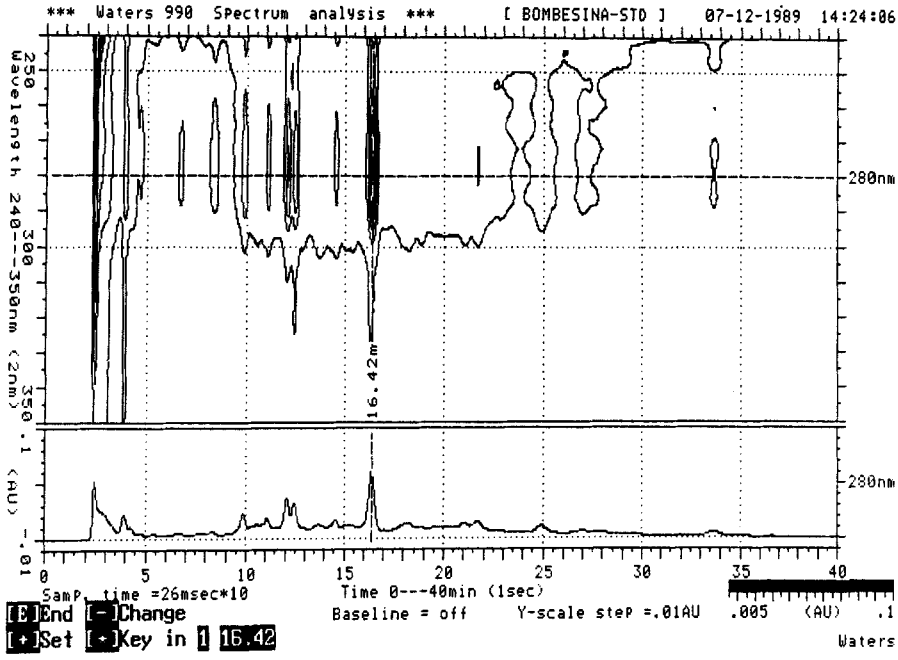

Fig. 2b

b) Spectrum analysis utilizing a photodiodes array M990 detector of the separation reported in Fig. 2a.

in the Materials and Methods section. A spectral analysis utilizing a photodiodes array M990 detector is reported in Fig. 1b.

Fig. 2a shows the results obtained after passing the crude material on a Protein-Pak SP 5PW (7.5 mm i.d. x 75mm) column eluted with a sodium acetate/sodium chloride linear gradient at pH 5. Capacity (Loading) tests on the same column were also performed injecting 2.5, 5 and 10 mg

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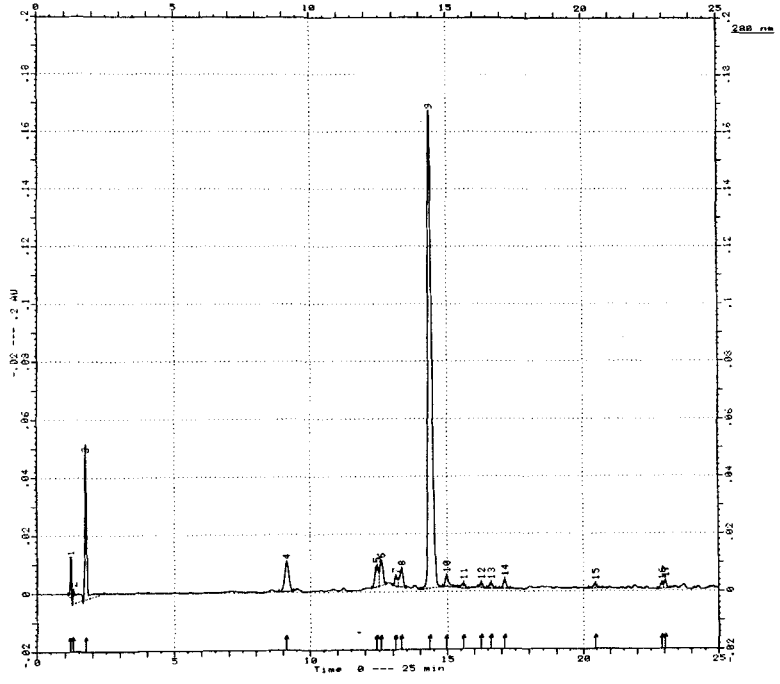

Fig. 3

a) RP-HPLC of the BN containing (16.42 min) major fraction obtained from ion-exchange purification of ≈ 10 mg of crude material (see Fig. 2b) on a 5μ Delta-Pak C-18, 100 Å, (3.9 mm i.d. x 300 mm) column. Buffers: 0.1% TFA in water = A; 0.1% TFA in acetonitrile = B. Gradient: linear from 20% to 40% B in 20 min. Flow-rate: 1 mL/min. Load: 25 μL. Sampling time: 28 msec. Y-Scale: 0.22 AU/FS. Wavelenght: 280 nm. Resolution: 2nm. Time range: 0-25 min. Interval: 1 sec. Paper speed: 10 mm/min.

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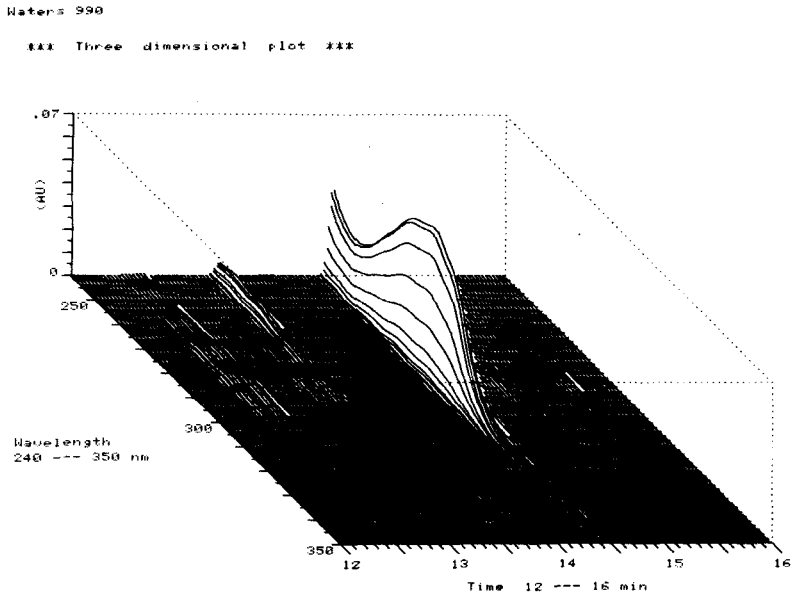

Fig. 3b

b) 3D-Plot of the spectrum analysis utilizing a photodiodes array M990 detector of the separation reported in Fig. 3a. Sampling time: 28 msec. Y-Scale: 0.07 AU/FS. Wavelenght: 240-350 nm. Resolution: 2nm. Time range: 12-16 min. Interval: 1 sec. Paper speed: 30 mm/min.

samples of the crude peptide. Fractions with a retention time of 16.42 min (Fig. 2b), corresponding to BN, were collected. In order to estimate the degree of purification achieved by the ion-exchange HPLC separation, the partially purified BN obtained from the 10 mg sample (see above) was

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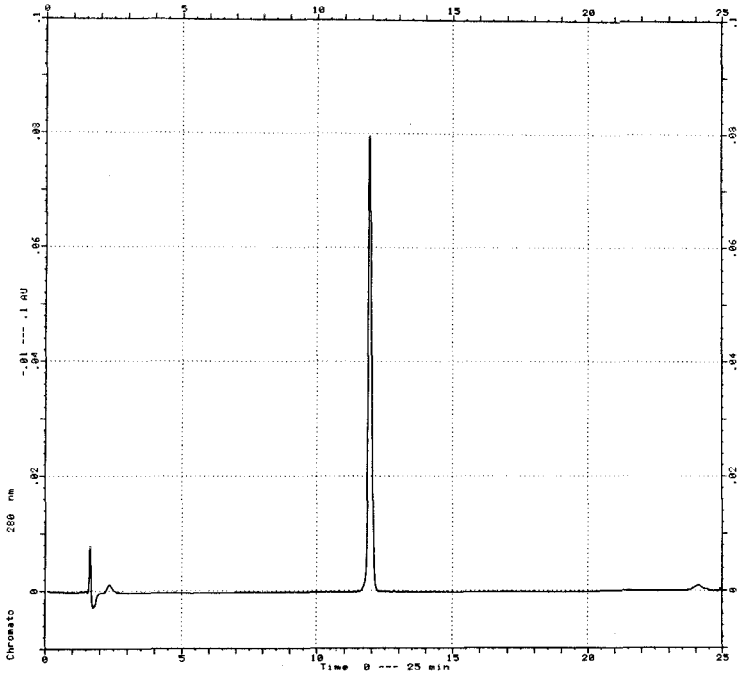

Fig. 4

a) RP-HPLC of the BN containing (14.14 min) major fraction of Fig. 3a, further rechromatographed on a 5µ Delta-Pak C-18, 100 Å, (3.9 mm i.d. x 150 mm) column. Buffers: 0.1% TFA in water = A; 0.1% TFA in acetonitrile = B. Gradient: linear from 20% to 40% B in 20 min. Flow-rate: 1 mL/min. Load: 25 µL. Sampling time: 26 msec. Y-Scale: 0.022 AU/FS. Wavelenght: 280 nm. Resolution: 2nm. Time range: 2.5-24 min. Interval: 1 sec. Paper speed: 10 mm/min. Under these experimental conditions BN elutes at 11.92 min.

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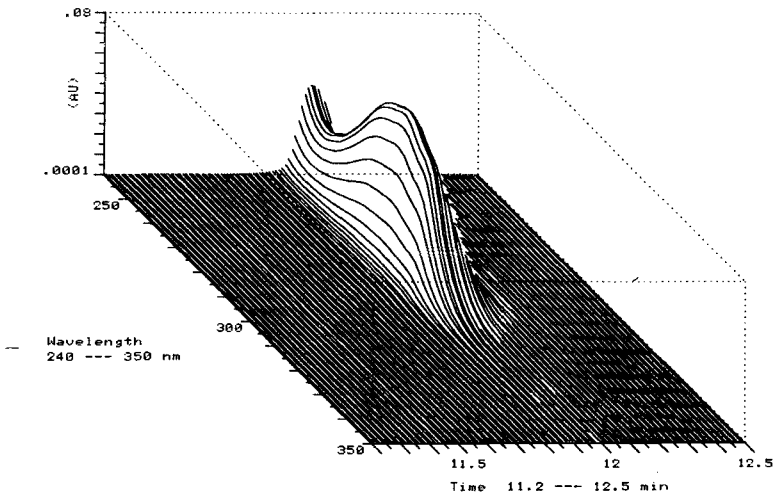

Fig. 4b

b) Off-scale of the separation reported in Fig. 4a

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analyzed by reverse-phase chromatography. The results are shown in Fig. 3a, while the corresponding spectrum, obtained with the photodiodes array detector is reported in Fig. 3b. A sample, further rechromatographed in the same reverse-phase conditions, yielded a product with purity ≥ 99% (Fig. 4a, b, c). The corresponding UV spectrum in the 200-340 nm range is reported in Fig. 4d.

The purified peptide was characterized by different criteria as described below.

Amino acid ratios in the acid hydrolysate of were (expected values in parentheses): His (1), 1.06; Arg (1),

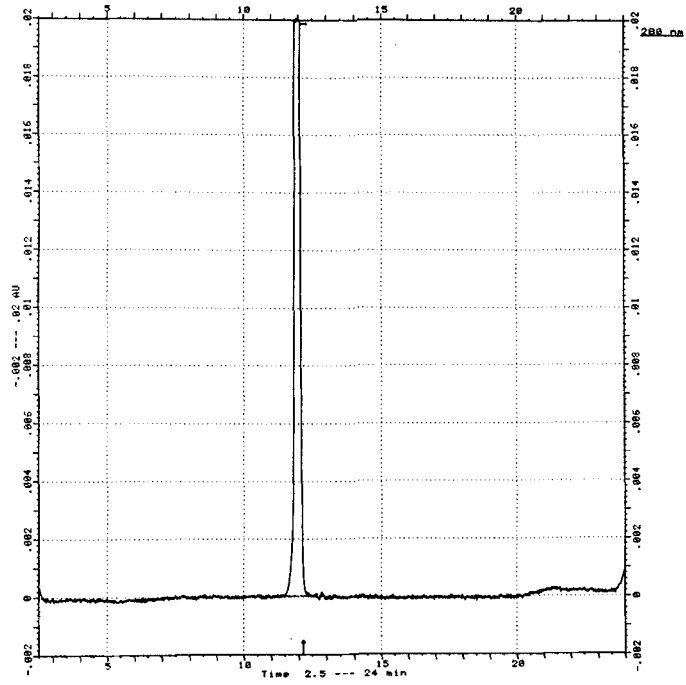

Fig. 4c

c) 3D-Plot of the spectrum analysis utilizing a photodiodes array M990 detector of the separation reported in Fig. 4a. Sampling time: 26 msec. Y-Scale: 0.0799 AU/FS. Wavelenght: 240-350 nm. Resolution: 2nm. Time range: 11.2-12.5 min. Interval: 1 sec. Paper speed: 80 mm/min.

1.04; Trp (1), n.d.; Asp (1), 1.11; Glu (3), 3.13; Gly (2), 2.02; Ala (1), 1.02; Val (1), 0.92; Met (1), 1.04; Leu (2), 2.04.

The biological activity, tested in the amylase release by pancreas disperse acini test, which is highly

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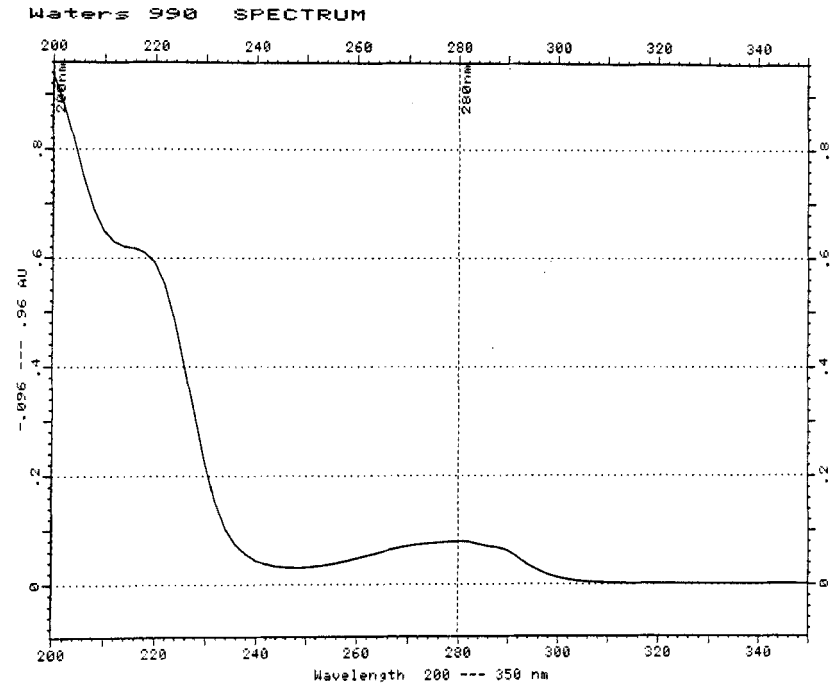

Fig. 4d

d) UV spectrum in the 200-350 nm range of the 11.92 min peak of purified BN (Fig. 4a). Sampling time: 26 msec. Y-Scale: 1.056 AU/FS. Wavelenght: 240-350 nm. Resolution: 2nm.

specific for BN (22), was comparable to that previously measured for authentic BN.

The purified peptide, submitted to FAB, gave good recovery of the molecular ion corresponding to the calculated molecular mass of 1620.

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DISCUSSION

The recent advance in solid-phase peptide synthesis, made possible by improved chemistry and technology, has been flanked by the continuous search for reliable fractionation methods for the effective purification of preparative amounts of synthetic peptides.

Bombesin (BN), a tetradecapeptide of formula <Glu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 with many interesting chemical and biological properties, which has previously served as a model to compare different strategies of peptide synthesis (23), has been selected for testing current purification technologies based on CCC and HPLC methods (24-25).

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It has been recently demonstrated that large amounts of crude synthetic peptides, including BN, can be rapidly and efficiently purified by CCC on the multi-layer coil planet centrifuge (26). However, the unquestionable advantages of this method characterized by great resolving power, high sample capacity and, especially, by the use of solvents only and no solid support, are so far limited by the complexity of the methodology. On the other hand, semi-preparative and preparative methods, based on the highly diffuse HPLC, have been hampered by the necessity of tedious procedures of multiple injections (25). As a matter of fact, the excellent resolution, typical of the analytical scale, is contrasted by the difficulty of maintaining high flow-rates and high pressure in the scaling up process. Furthermore, in column

chromatography, loss of sample and limited reusability of the solid support have always to be taken into consideration. Nevertheless, recent improvements both in the materials and instrumentation, seem to have overcome most of these drawbacks.

In this study, we have performed a purification scheme based on a combination of ion-exchange and reverse-phase HPLC that allows the rapid and effective purification of the highly heterogeneous BN generated by solid-phase methods. In particular, ion-exchange chromatography on cationic Protein-Pak SP 5PW analytical columns has allowed an easy preliminary purification of up to 50 mg of crude material with quantitative recovery of the target molecule and good reusability of the column. Further purification by RP-HPLC on C-18 columns gave salt-free BN samples with purity ≥ 99% which were easily liophylized and characterized by different physico-chemical and biological criteria including amino acid analysis, after acid hydrolysis, FAB, TLC and the amylase release test which is highly specific for BN. Large scale purification (data not shown) of 0.5-1 g of crude material have been conveniently achieved by a similar purification scheme using preparative glass columns.

The present approach, which is currently utilized also for molecules related to BN, e.g. analogs, antagonists etc., seems to be particularly promising in view of the increasing demand of highly purified peptides, which are used to study their structural and physico-chemical properties (27-28) and may be useful not only to further explore the biological

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role and mode of action but, hopefully, also for clinical applications.

Acknowledgement

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