Journal of Chromatography, 296 (1984) 181–188
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 357

PURIFICATION OF RADIOIODINATED SOMATOSTATIN-RELATED PEPTIDES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

In order to set up specific radioimmunoassays for the two N- and C-terminal tetradecapeptides of somatostatin-28 the peptides somatostatin SS_{14} and SS_{28} and the somatostatin by-products 1-Tyr-SS_{14}, 11-Tyr-$_{14}$ and desaminotyrosyl-β-alanine fragment (1→14) of SS_{28} were radioiodinated by the chloramine-T or Bolton–Hunter techniques. Reversed-phase high-performance liquid chromatography was shown to be a very efficient and reliable method for the purification of different radioactive reaction media. The corresponding labelled peptides were tested for their relative immunological (radioimmunoassay) and biological (binding studies) properties.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) has already been reported as an accurate method for the purification of somatostatin from several tissues and species[1–5] and for the purification of synthetic material[6–8]. Its elution under different conditions has been studied[9].

We report here the labelling procedure for several synthetic somatostatin compounds: 1-Tyr-SS_{14}, 11-Tyr-SS_{14}, desaminotyrosyl-β-alanine fragment (DT-β-Ala F) (1 → 14) of SS_{28} using the chloramine T. method. F (1 → 14) of SS_{28} and SS_{28} were iodinated by conjugation according to the Bolton and Hunter procedure. The further purification of the iodinated peptide was achieved by RP-HPLC on a μBondapak column.

EXPERIMENTAL

Peptides

Somatostatin-28[6] was a gift from Prof. E. Wünsch (Munich, F.R.G.). Fragment (1 → 14) of SS_{28} and its by product DT-β-Ala F (1 → 14) were synthesized by Dr. M. Gemeiner and Prof. L. Moroder (the synthesis will be described wlsewhere) in Prof. E. Wünsch's Laboratory (Max Planck Institut, Munich, F.R.G.). 1-Tyr-SS_{14} and 11-Tyr-SS_{14} were purchased from Bachem (Bubendorf, Switzerland).

Chemicals

All reagents were of analytical-reagent grade. Triethylamine (puriss) and acetonitrile (for spectroscopy grade) were supplied by Fluka (Buchs, Switzerland). Orthophosphoric acid (G. R. grade) and chloramine T were purchased from Merck (Darmstadt, F.R.G.). Iodine-125, IMS-30 carrier-free in NaOH solution and ^{125}I-labelled Bolton–Hunter reagent (*ca.* 2 Ci/μmole) were obtained from Amersham France. Tyrosine and glycine were from a Sigma amino acid calibration kit.

HPLC

HPLC was performed on a μBondapak c_{18} column (30 × 0.39 cm I.D.) at room temperature. The mobile phase was composeed of 0.25 *N* triethylammonium phosphate (TEAP) buffer (pH 3.5)[10] combined with acetonitrile. A Waters Assoc. liquid chromatograph was used, consisting of a U6K injector, a 6000 A pump, a Schoeffel Model 770 multi-wavelenght detector and an Omniscribe chart recorder associated with an LKB 2112 Redirac fraction collector.

The absorbance at 210 nm was continuously recorded and the radioactivity of 500-μl fractions was determined by counting an aliquot of each fraction in a Model PGP Prias automatic gamma counter (Packard, Downers Grove IL, U.S.A.). After each purification of radioiodinated polypeptide, an injection of the corresponding unlabelled molecule was made in order to locate the elution position of the unreacted peptide. For UV and radioactive peak identification, all reagents or media blanks were chromatographed.

Radioiodination processes

Variants of the chloramine T method initially described by Greenwood *et al.*[11] were used for 1-Tyr-SS_{14} and 11-Tyr-SS_{14} iodination and for DT-β-Ala F (1 → 14) of SS_{28}.

For the preparation of [^{125}I]-1 and -11-Tyr-SS_{14} the experimental conditions were as follows: 40 μl of 0.5 *M* phosphate buffer (pH 7.5); 1 mCi (10 μl) of $Na^{125}I$ and 2.4 nmole of 1- and 11-Tyr-SS_{14} (10 μl); 10 μl of 0.5 μg/μl aqueous chloramine-T solution were added in four steps at 10-sec intervals, followed by 120 μl of tyrosine (2 μg/μl) dissolved in 0.5 *M* phosphate buffer pH 7.5–1 *M* NaOH (80:20). Tyrosine solution was added to stop the reaction by fixing the excess of free iodine.

For iodination of DT-β-Ala F (1 → 14) of SS_{28} the same proportions and same reagents were used, except that 10 μl of aqueous sodium metabisulphite solution (0.5 μg/μl) were added to stop the reaction instead of tyrosine. An isotopic dilution of iodine was made with 400 μg of $Na^{127}I$ in 200 μl.

Bolton and Hunter procedure. The free amino groups of SS_{28} and of F (1 → 14) of SS_{28} were condensed with iodinated Bolton–Hunter reagent[12] at 4°C. The reagent (500 μCi solution in benzene) was rapidly evaporated under a nitrogen stream. To the residue was added 15 μl of 0.05 *M* sodium borate buffer (pH 8.4) and 0.5 nmole of F (1 → 14) of SS_{28} in 10 μl of borate buffer. After incubation for 30 min the reaction was stopped with 200 μl of 0.2 *M* glycine solution in borate buffer.

Biological studies

The highly radioactive fractions were stored in dilute form (10^6 cpm/ml) at −30°C in 20 m*M* EDTA–12% BSA–200 μg/l bacitracin–0.25 *N* TEAP buffer solution

(pH 3.5). Their ability to bind specifically to receptors of isolated acini was studied according to the method of Sankaran *et al.*[13]. The radioimmunological properties of these fractions were analysed by association with two different antibodies directed against the N- and C-terminal parts of SS_{28}.

RESULTS

Purification of [^{125}I]-1-Tyr-SS_{14} and [^{125}I]-11-Tyr-SS_{14}

Fig. 1 illustrates the UV absorbance and radioactive elution profiles obtained after injection of labelled 1-Tyr-SS_{14} (Fig. 1A) and 11-Tyr-SS_{14} (Fig. 1B). The isocratic conditions that gave the best separation of the labelled molecules from unreacted peptide without too much spreading of the monoiodinated peptide were 73:27 and 77:23 TEAP–CH_3CN, respectively. These conditions were selected after varying the elution buffer and testing the immunoreactivity of the eluted material. The unreacted peptides were eluted at 14 and 10 min, respectively. Their peak areas indicate that a large part of the peptide did not react during the iodination process chosen. A clear but very small absorbing area is present under the main radioactive component.

The radioactive elution profiles showed two main areas; that close to the void volume corresponds to an excess of iodine, unreacted or coupled to tyrosine. The monoiodinated peptides were eluted at 11 and 9 V_o. Late-emerging radioactive species that were found to be inactive on immunological or biological tests were not investigated further.

As each radioactive material eluted in the main radioactive peak corresponds to a UV-absorbing product, direct quantification of the specific activity of the tracer is possible. For this purpose a calibration graph was constructed using a buffer in which the acetonitrile concentration had been reduced in order to elute the unreacted peptide with a retention time close to 20 min. Using this calibration graph the specific activity of the tracer gave a mean value of 700 μCi/nmole.

Purification of [^{125}I]desaminotyrosyl fragment (1 → 14) of SS_{28}

The desaminotyrosyl fragment (1 → 14) of SS_{28} is a derivative specially developped for labelling an N-terminal fragment of SS_{28} and to set up a specific radioimmunoassay (RIA) of somatostatin 28 that does not react with SS_{14}.

Fig. 2A shows a typical UV absorbance profile given by a semi-purified preparation of this peptide when TEAP–CH_3CN (90:10) was used. The material used for iodination corresponds to the 12 V_o peptide eluted from this HPLC system. The elution power of the buffer used for the purification of its labelling medium (Fig. 2B) was increased by increasing the acetonitrile concentration to 14%. Under these conditions, the peptide was eluted between 2.5 and 3 V_o. The radioactive materials were eluted as four species with relative percentages of 14.5 ± 4 (t_R 6.5 min) 52.5 ± 6 (t_R 12 min), 18.1 ± 4 (t_R 18 min) and 14.5 ± 3 (t_R 30 min).

Radioactivity recovery studies were carried out, chromatography being performed with a buffer containing 50% of acetonitrile. This buffer eluted no additional radioactive product. The chromatographic recovery was then determined by an analytical run (samples injected corresponding to 1/100 of the labelling medium) and under these conditions the recovery was 95 ± 15%.

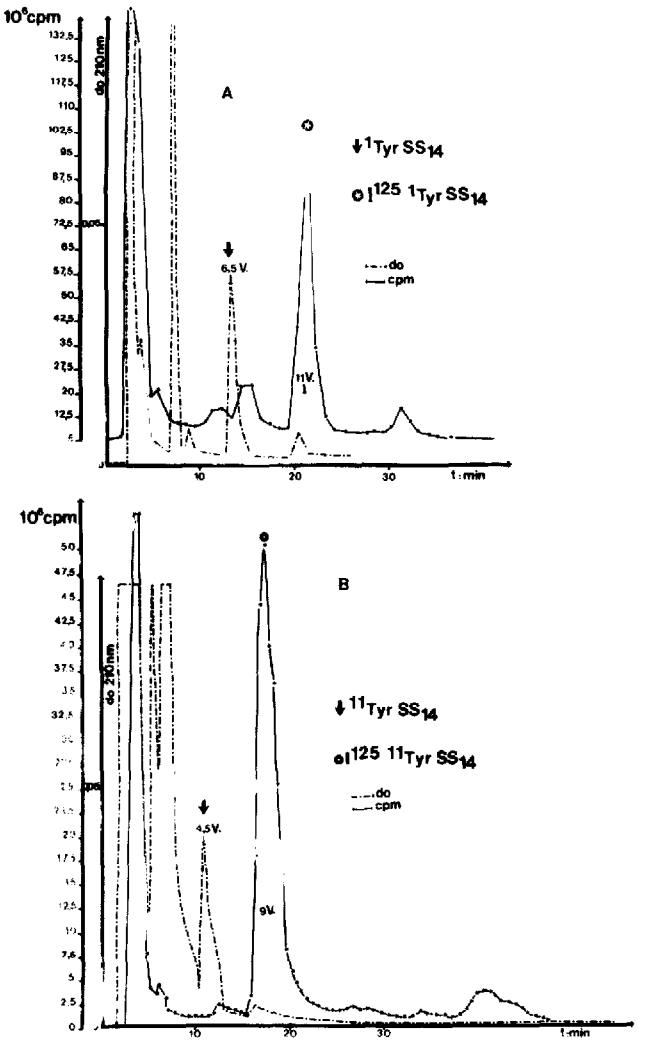

Fig. 1. (A) Purification of [^{125}I]-1-Tyr-SS_{14} on μBondapak C_{18}. Elution buffer, TEAP–CF_3CN (73:27); flow-rate, 1 ml/min; fraction volume, 0.5 ml; void volume, V_o = 2.2 ml. - - - - -, Absorbance at 210 nm; ——, pm/fraction. Arrow, elution position of unreacted 1-Tyr-SS_{14}; star, elution position of the tracer. (B) Purification of [^{125}I]-11-Tyr-SS_{14} on μBondapak C_{18}. Elution buffer, TEAP–CH_3CN (77:23); flow-rate, 1 ml/min; fraction volume, 0.5 ml, void volume, V_o = 2.2 ml. - - - - -, Absorbance at 210 nm; ——, cpm/fraction. Arrow, elution pposition of unreacted 11-Tyr-SS_{14}; star, elution position of the tracer.

Purification of ^{125}I-labelled Bolton–Hunter conjugates of SS_{28} and its N-terminal F (1 → 14) fragment

Owing to the high hydrophobic characteristics given by the conjugation of a small peptide to the Bolton–Hunter reagent, a step gradient elution is necessary to recover the different components of the labelling medium. For SS_{28} (Fig. 3B) a step gradient from 25 to 50% acetonitrile was chosen; the duration of chromatography in the first solvent was sufficient to provide complete elution of the unreacted SS_{28}.

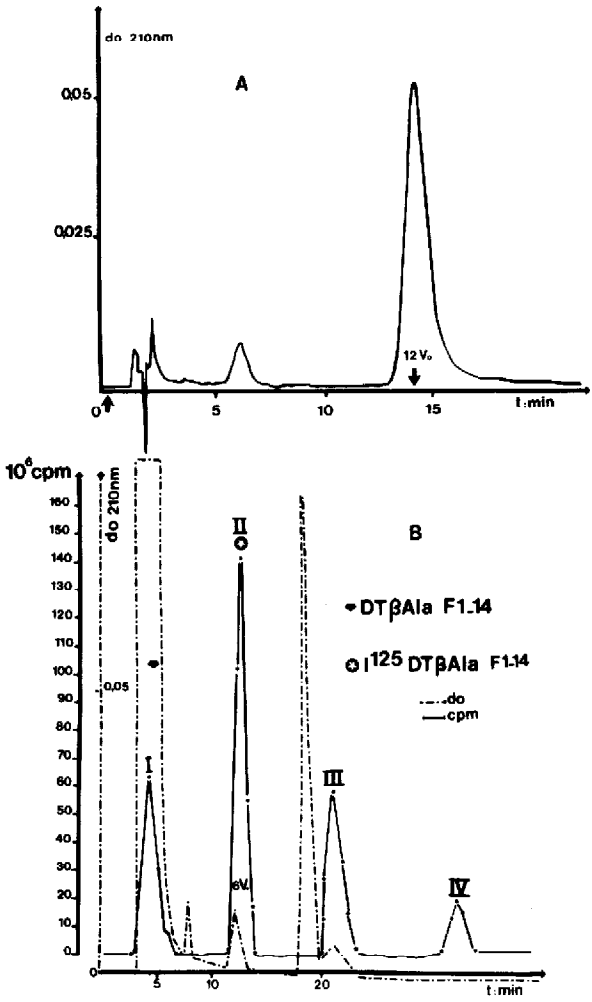

Fig. 2. (A) Purification of the DT-β-Ala (1 → 14) of SS_{28} by RP-HPLC. Elution buffer, TEAP–CH_3CN (90:10). Arrow, elution position of the collected material. (B) Purification of [^{125}I]-DT-β-Ala F (1 → 14) of SS_{28}. Elution buffer, TEAP–CH_3CN (86:14); flow-rate, 1 ml/min; fraction volume, 0.5 ml. - - - - -. Absorbance at 210 nm; ——, cpm/fraction. Arrow, elution position of DT-β-Ala F (1 → 14) of SS_{28}; star, elution position of the radioactive products selected as tracer.

With the N-terminal polar part of SS_{28} an 7.5% higher initial concentration of acetonitrile was added in order to discriminate better the radioactive components from the unreacted peptides (Fig. 3A).

Biological and radioimmunological properties of the iodinated peptides

For antibody association studies, the purified products (*ca.* 3–5 fmole) were incubated with an appropriate dilution of antibody to obtain a *ca.* 50% association at equilibrium; the respective IC_{50} (*i.e.*, the amount of peptide added in the test to displace 50% of bound material) for SS_{14} and SS_{28} were 0.025 p*M* ([^{125}I]-1-Tyr-SS_{14}), 0.2 p*M* ([^{125}I]-11-Tyr-SS_{14}) and 0.25 p*M* {[^{125}I-DT-β-Ala F (1 → 14) of

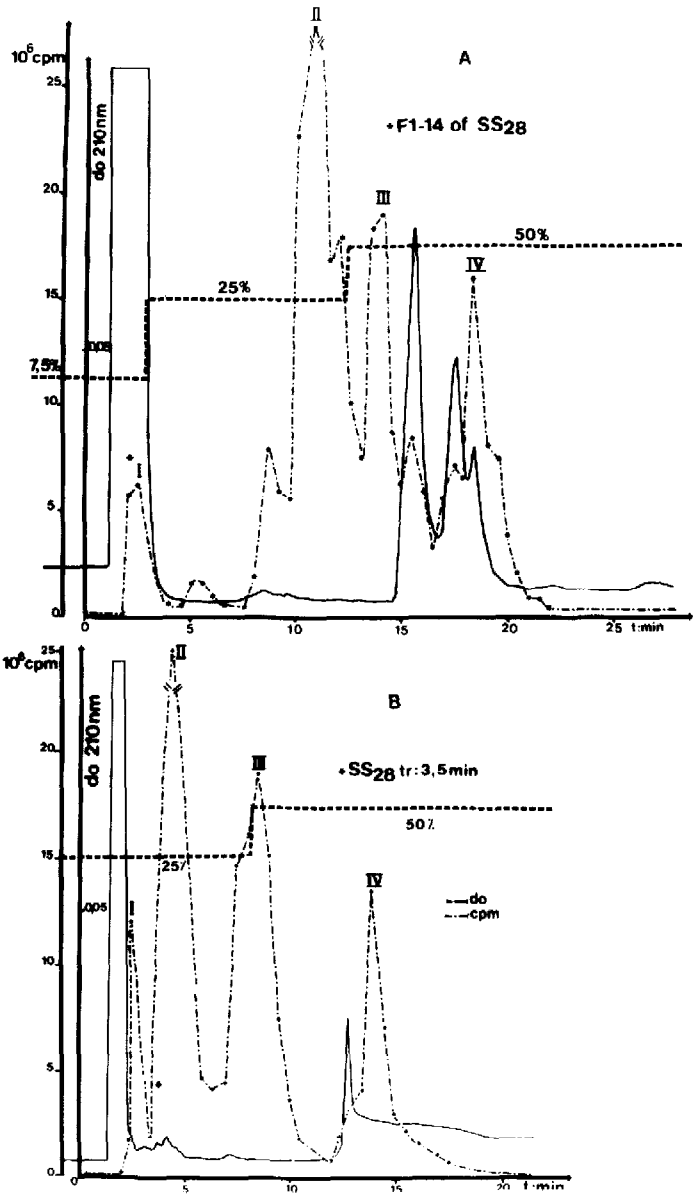

Fig. 3. (A) Purification of the ^{125}I-labelled Bolton–Hunter fragment (1 → 14) of SS_{28} conjugates by step gradient elution. Elution buffers, TEAP with increasing amounts of CH_3CN (7.5, 25 and 50%; flow-rate, 2 ml/min; fraction volume, 1 ml. Arrow, elution position of F (1 → 14) of SS_{28}. (B) Purification of the I^{125}-labelled Bolton–Hunter fragment of SS_{28} conjugates. Step gradient elution performed with TEAP with acetonitrile concentrations of 25 and 50%; flow-rate, 2 ml/min; fraction volume, 1 ml. Arrow, elution position of SS_{28}.

SS_{28}}. For the last iodinated compound only SS_{28} acts on the antibody binding.

Specific and saturable binding was obtained on guinea pig pancreatic acini membranes (K_d = 0.31 ± 0.05 nM)[14].

DISCUSSION

RP-HPLC systems based on isocratic or step gradient elution studied for several iodinated somatostatin derivatives. For molecules in which a tyrosyl residue was included in order to provide direct incorporation of iodine by classical methods, HPLC improved the purification of the labelling medium of these molecules. The technique allows the rapid recovery of the tracers and the direct calculation of their specific activity. As the method permits complete separation of the unreacted peptide, we choose to use experimental conditions under which a small amount of the peptide is labelled in order to minimize the amount of diiodinated peptide and also to reduce the degradation of the tryptophan residue during oxidation. In each instance the iodinated peptides were more strongly bound to the column phase than the unlabelled peptide, which is terms of polarity is unexpected. The iodinated peptides probably undergo supplementary changes that affect their retention on the C_{18} columns.

When conjugation with the Bolton–Hunter reagent is performed, the change in hydrophobicity due to this coupling is still larger. Further, the HPLC system allows the separation of several radioactive species, probably corresponding to the different amino groups that can react with the Bolton–Hunter reagent and to several combinations of these multiple incorporation sites.

Unfortunately, a complete study of this phenomenon has not been possible, as even the smallest incorporation with this reagent results in a product that appears to have a very low affinity for the antibodies and receptors used. This indicates that the reaction on the free amino group is highly detrimental to the model we have used.

ACKNOWLEDGEMENTS

The authors especially thank Mr. C. Mora and Mrs. A. M. Remaury for technical assistance.

REFERENCES

1 J. Spiess, J. Rivier, J. A. Rodkey, C. D. Benneth and W. Vale, *Proc. Nat. Acad. Sci. U.S.*, 76 (1979) 2974.
2 L. Pradayrol, J. A. Chayvialle, M. Carlquist and V. Mutt, *Biochem. Biophys. Res. Commun.*, 83 (1978) 701.
3 A. V. Schally, W. Y. Huang, R. C. C. Chang, A. Arimura, T. Redding, R. Millar, M. Hukapiller and L. E. Hood, *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 4489.
4 F. Esch, P. Böhlen, N. Ling, R. Benoit, P. Brazeau and R. Guillemin, *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 6827.
5 P. Böhlen, P. Brazeau, F. Esch, N. Ling and R. Guillemin, *Regulatory Peptides*, 2 (1981) 359.
6 E. Wünsch, L. Moroder, M. Gemeiner, E. Jaeger, A. Ribet, L. Pradayrol and N. Vaysse, *Z. Naturforsch. B*, 35 (1980) 911.
7 N. Ling, F. Esch, D. Davis, M. Mercado, M. Regno, P. Bohlen, P. Brazeau and R. Guillemin, *Biochem. Biophys. Res. Commun.*, 95 (1980) 945.
8 C. Tronquet, J. P. Guimbard and F. Paolucci in G. Rosselin, P. Fromageot and S. Bonfils (Editors), *Hormone Receptors in Digestion and Nutrition*, Elsevier North Holland Biomedical Press, Amsterdam, New York, 1979, p. 89.
9 M. Abrahamsson and K. Gröningsson, *J. Liq. Chromatogr.*, 3 (1980) 495.
10 J. E. Rivier, *J. Liq. Chromatogr.*, 1 (1978) 343.
11 F. C. Greenwood, W. M. Hunter and J. S. Glover, *Biochem. J.*, 89 (1963) 114.

12 A. E. Bolton and W. M. Hunter, *J. Endocrinol.*, 55 (1972) 30.
13 H. Sankaran, I. D. Goldfine, C. W. Deveney, K. Y. Wong and J. A. Williams, *J. Biol. Chem.*, 255 (1980) 1849.
14 D. Taparel, J. P. Esteve, Ch. Susini, N. Vaysse, D. Bals, G. Berthon, E. Wünsch and A. Ribet, *Biochem. Biophys. Res. Commun.*, 115 (1983) 827.