*Journal of Chromatography*, 447 (1988) 341-349
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 538

# TYROSYLATION AND PURIFICATION OF PEPTIDES FOR RADIOIODINATION

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(First received November 25th, 1987; revised manuscript received February 18th, 1988)

## SUMMARY

A simple method for tyrosylation, purification and subsequent radioiodination of peptides which lack suitable acceptor groups for iodine substitution is presented. The reagent, *tert.*-butyloxycarbonyl-L-tyrosine N-hydroxysuccinimide ester, was used for conjugation to the amino groups of peptides. The derivatization was performed with relatively large amounts of reagents to ensure quantitative reactions. The derivatized peptides were purified by reversed-phase high-performance liquid chromatography or by gel filtration. Subsequent radioiodination was performed with sodium [$^{125}$I]iodide and the sparingly soluble tetrachlorodiphenylglycouril as the oxidative agent to minimize possible oxidative damage to the peptides. The radiolabelled peptides were subsequently purified by isocratic high-performance liquid chromatography.

## INTRODUCTION

Radioiodination of peptides is the most commonly used labelling method for the generation of tracers for immunoassays and receptors studies. The iodination is generally performed by chemical substitution of hydrogen in tyrosyl or histidyl residues using oxidative agents such as chloramine-T[1,2], lactoperoxidase[3] or iodogen[4,5]. Techniques have also been developed to protect the peptide from oxidative damage, through separate labelling of a reactive precursor, which is extracted from the iodination mixture and then conjugated to the peptide under mild conditions. The conjugation methods are also useful for the labelling of peptides lacking tyrosyl or histidyl residues. The conjugation reagents include N-succinimidyl 3-(4-hydroxyphenyl)propionate (Bolton–Hunter reagent)[6], methyl-*p*-hydroxybenzimidate[7], diazotized aniline[8] and *tert.*-butyloxycarbonyl(t-BOC)-L-tyrosine N-succinimidine ester (Associan reagent)[9].

Radioiodinated forms of at least the Bolton–Hunter reagent are available commercially. In the reaction with labelled derivatizing reagent, only small, but nevertheless expensive amounts of radiolabelled conjugation reagent and peptide are used. Thus, success is dependent on the absence of chemical scavengers interfering with

0021-9673/88/$03.50 

the conjugation reagent or peptide. In organic chemistry laboratories highly purified solvents, etc., are generally available; however, most radioiodinations are performed in physiological and clinical laboratories. We present a simple method for tyrosylation and purification of relatively large amounts of peptides for subsequent routine radioiodination. The method offers an economical alternative to iodination with radioiodinated conjugation reagents. The problems of oxidative damage to the peptide during the iodine substitution are circumvented by using chloroglycouril as the non-soluble oxidative agent, and by subsequent purification of the radioiodinated peptides by isocratic high-performance liquid chromatography (HPLC).

## MATERIALS AND METHODS

### *Peptides*

Bovine pancreatic polypeptide (BPP) was a generous gift from Dr. Ronald E. Chance (Eli Lilly, Indianapolis, IN, U.S.A.). Somatostatin-28 (1-12-fragment) was obtained from Peninsula Laboratories (St. Helens, U.K.) by Drs. F. Baldissera and J. J. Holst. The $Cys_0$-preproneuropeptide-$Y_{83-88}$ (Cys-Thr-Glu-Asn-Val-Pro-Arg) was synthesized by Cambridge Research Biochemicals (Cambridge, U.K.). Dog pancreatic eicosapeptide was isolated from the duodenal part of the pancreas by acid–ethanol extraction, ether precipitation, gel filtration chromatography and HPLC as described[10,11].

### *Tyrosylation*

Around 50 nmol of BPP, 75 nmol of somatostatin-28 (1–12-fragment) and 1.5 μmol of $Cys_0$-preproneuropeptide-$Y_{83-88}$ were conjugated, whereas only 5 nmol of the eicosapeptide were used. The peptides were dried under vacuum and reconstituted in 200 μl of 0.1 *M* N-ethylmorpholine, analytical grade (Serva Feinbiochemica, Heidelberg, F.R.G.), adjusted to pH 8.0 with glacial acetic acid (Merck, Darmstadt, F.R.G.). The tyrosylation reagent was added as 50 μl of a 10 mmol/l solution of t-BOC-L-tyrosine-N-hydroxysuccinimide ester, research grade (Serva), in dimethylformamide, Sequanal Grade (Pierce Chemical, Rockford, IL, U.S.A.). The reaction time was usually 15 min, however in some cases the reaction mixture was analysed by HPLC after various periods. The derivatized peptides were purified either by HPLC or by gel filtration on a 25 cm × 1 cm Bio-Gel P-2 column, eluted at room temperature with 0.05 mol/l phosphate buffer, pH 7.5. The HPLC system used for analytical and preparative purposes consisted of a 25 cm × 0.4 cm reversed-phase Nucleosil 300-5 $C_{18}$ column, 5-μm particles and pore diameter 300 Å. The packing material was obtained from Macherey Nagel (Düren, F.R.G.) and packed into stainless-steel columns (Knauer, Berlin, F.R.G.). The chromatography was performed at 50°C on an Hewlett-Packard 1090 liquid chromatograph with 0.1% (v/v) trifluoroacetic acid (Art. No. 8262, Merck) in water as the aqueous phase and acetonitrile (chromatography grade, Merck) as the organic solvent.

The protecting BOC group was removed from the derivatized, HPLC-purified peptides by treating the dried peptide either with 50 μl of 99% formic acid (Art. No. 264, Merck) for 1 h at room temperature or with undiluted trifluoroacetic acid (Pierce). The solvents were removed under vacuum and the deprotected, derivatized peptide was reconstituted in 50 μl of 0.1% (v/v) trifluoroacetic acid before purification by HPLC.

*Sequence determination*

The solvent was removed under vacuum from the derivatized and deprotected which was reconstituted in 60 μl of 0.1% (v/v) acetic acid, and subjected to automated sequence analysis by sequential Edman degradation on an Applied Biosystem 470A gas-phase sequenator. The MHTFA1 program of M. Hunkapillar which is a modification of that of Hunkapillar *et al.*[12] of 1983, and available from Applied Biosystems (Foster City, CA, U.S.A.), was used. All chemicals were obtained from Applied Biosystems. The characterization of the phenylthiohydantoin derivatives of amino acids was performed on an Hewlett-Packard 1090 liquid chromatograph with a 25 cm × 0.45 cm CN column with 5-μm particles (IBM Instruments, CT, U.S.A.) and a sodium acetate–acetonitrile gradient elution system as described[13]. Before analysis, the samples from the sequenator were methylated by treatment with acidified methanol (1 *M* hydrochloric acid in methanol, Applied Biosystems) for 10 min at 50°C. Aminobutyric acid was used as an internal standard during HPLC, for correction and for quantitation of the amino acid derivatives.

*Amino acid analysis*

For hydrolysis, HPLC-purified derivatized peptides were dried under vacuum in 50 mm × 6 mm Culture Tubes (Corning, Stone, U.K.). The tubes were placed in PICO-TAG reaction vials (Waters, Milford, MA, U.S.A.) containing 200 μl of 6 *M* hydrochloric acid with 10 μl phenol. The hydrolysis was performed at 110°C under vacuum for 20–24 h. The excess of hydrochloric acid was removed under vacuum. The free amino acids were analysed as phenylthiocarbamyl derivatives by reversed-phase HPLC on a Merck Supersphere column $C_8$, 25 cm × 0.4 cm as previously described[14].

*Iodination*

The derivatized pancreatic eicosapeptide was iodinated with carrier-free $Na^{125}I$ (Amersham, Little Chalfont, U.K.) using the oxidation reagent, 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Serva) as described[4]. The chloroglycouril (3.7 mg) was dissolved in 2.0 ml dichloromethane (Sigma, St. Louis, MO, U.S.A.) and the inside of an Eppendorff test-tube (Sarstedt, Nümbrecht, F.R.G.) was coated with 20 μl of this solution by gently turning the tube while the solvent evaporated. The derivatized eicosapeptide (1.5 nmol) was dried under vacuum, reconstituted in 40 μl phosphate buffer, pH 7.38, precision buffer solution (Radiometer, Copenhagen, Denmark) and added to the coated tube. The salt $Na^{125}I$ (1 mCi) was added and the tube was kept on ice. After 5 min, 50 μl of the HPLC solvent (28% acetonitrile in 0.1% trifluoroacetic acid in water) were added before purification on a Nucleosil 300-5 $C_{18}$ column, 25 cm × 0.4 cm, eluted isocratically at 50°C with a flow-rate of 1.0 ml/min. Fractions of 0.5 ml were collected and aliquots of 10 μl counted in a gamma counter.

*Test of radioiodinated, derivatized peptide*

The derivatized and radioiodinated eicosapeptide was tested for reaction with antisera in radioimmunoassay. Antibodies, standards and labelled peptide were incubated for 48 h at 4°C in tubes containing a total of 1.5 ml of 0.2 mol/l phosphate buffer, pH 7.5, with 2.5 mg/l bovine serum albumin (Sigma). The separation of free and bound peptide was performed by addition of 0.5 ml of a slurry of activated charcoal

(Sigma) 2 g/100 ml in assay buffer supplemented with 10% outdated plasma, and centrifugation (10 min at 4000 *g*) after incubation for 20 min at room temperature.

## RESULTS AND DISCUSSION

### *Reaction time*

Pancreatic polypeptide was used to investigate the reaction time. Around one third of the peptide had reacted within 30 s (the time required to inject the sample in HPLC) using 50 nmol of peptide in 0.25 ml and a ten-fold excess of derivatization reagent, Fig. 1a. After a few minutes the reaction was almost complete while the conjugation reagent had started to hydrolyse. The reagent was completely hydrolysed at 40 min, Fig. 1b. When the reagent was dissolved and kept in acetonitrile, no change in elution position, corresponding to the solid arrow in Fig. 1a, was detected even after several hours. However, in 50% acetonitrile in water, the reagent disappeared gradually and a major hydrolysis product appeared with an elution position corresponding to the open arrow in Fig. 1b (data not shown). A reaction time of 15 min was chosen.

### *Purification*

With relatively apolar peptides, *e.g.*, pancreatic polypeptide (PP), the purifica-

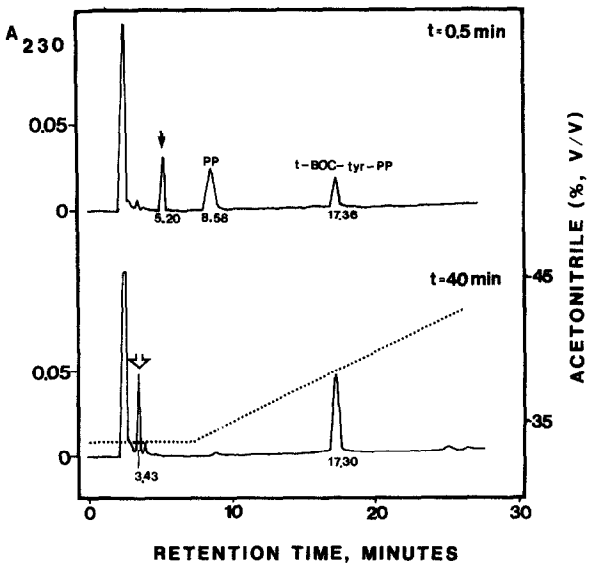


Fig. 1. Derivatization of pancreatic polypeptide (a 36-amino acid, relatively apolar peptide) and hydrolysis of the derivatization reagent. HPLC profile of the reaction mixture after 0.5 min of incubation, upper panel, and after 40 min, lower panel. The elution positions of underivatized pancreatic polypeptide, PP, and of the derivatized peptide, t-BOC-Tyr-PP, are indicated. The solid arrow indicates the elution position of freshly dissolved t-BOC-L-Tyr-N-hydroxysuccinimide ester and the open arrow that of the major hydrolysis product of this reagent. The column (25 cm × 0.4 cm) was packed with Nucleosil $C_{18}$ with a particle size of 5 μm, a pore size of 300 Å and eluted with 0.1% TFA–water and a gradient of acetonitrile as indicated by the dotted line.

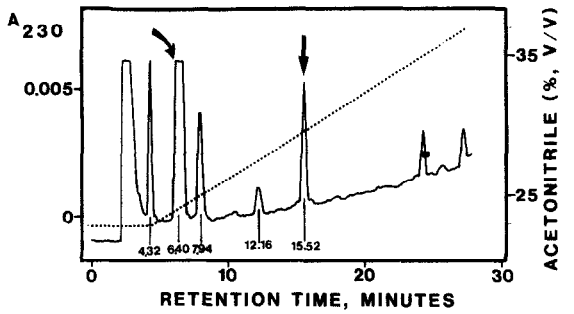


Fig. 2. HPLC purification of derivatized pancreatic eicosapeptide (a relatively polar peptide). Profile of the reaction mixture after 35 min of incubation. In this system the derivatization reagent is eluted after 12.16 min and the major hydrolysis product after 6.40 min; the peaks at 4.32 and 7.94 min are also present when no peptide is added to the reaction mixture. The elution position of the underivatized eicosapeptide is indicated by the curved arrow. The peak at 15.52 min is due to the derivatized eicosapeptide, as demonstrated by amino acid analysis.

tion by HPLC is straightforward since both the normal peptide and the derivatized peptide are eluted later than the conjugation product and its hydrolysis products. However, smaller and more polar peptides may coelute with the reagents which was the case with the canine pancreatic eicosapeptide as shown in Fig. 2. This figure demonstrates that the interpretation of the HPLC elution profile is no longer simple

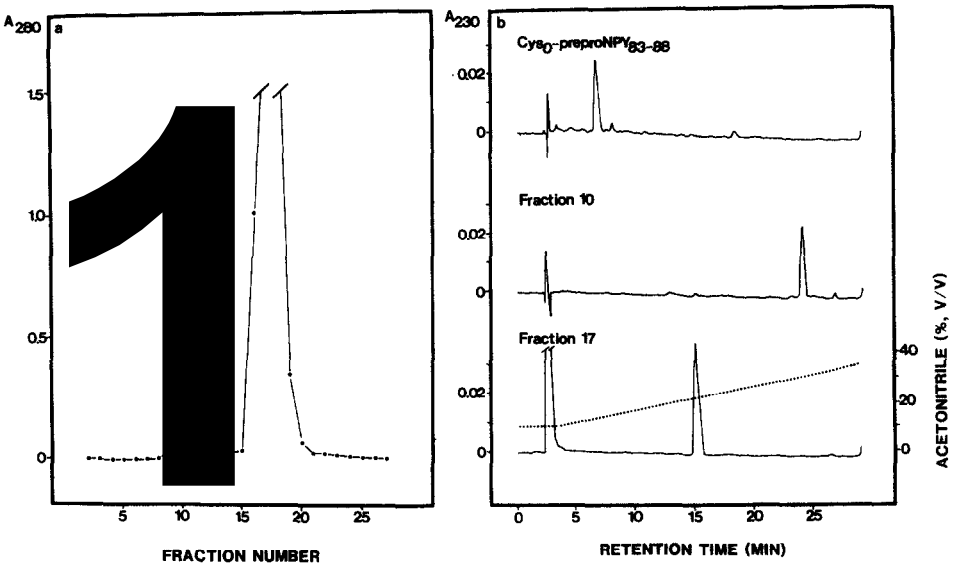


Fig. 3. Purification by gel filtration of a small derivatized peptide, $Cys_0$-preproneuropeptide-$Y_{83-88}$. (a) Gel filtration of the reaction mixture separated on a Bio-Gel P-2 column (25 cm × 1 cm) eluted with 0.05 mol/l phosphate buffer, pH 7.5 at room temperature. Fractions 9–13 contain the derivatized peptide. (b) HPLC of the underivatized peptide (upper), purification of gel filtration fraction 10 containing the derivatized, more apolar peptide eluted after 24 min (middle) and profile of gel filtration fraction 17 containing the hydrolysed reagent eluted after 15 min and solvents eluted close to the injection artefact (lower). Column and solvents as in Fig. 1.

when a small amount of peptide is derivatized. We used relatively large amounts of conjugation reagent, 2 mmol/l, to avoid quenching of the reaction by interfering contaminants.

The most generally applicable purification method is gel filtration on, *e.g.*, a Bio-Gel P-2 column. In Fig. 3a is shown the purification of derivatized $Cys_0$-preproneuropeptide-$Y_{83-88}$ by gel filtration. Through this "desalting" procedure, the reagent and solvent of the reaction mixture are eluted in the total volume of the column and the oligopeptide in the void volume. When desalting is used, it is recommended to test the gel-filtered reaction product by analytical HPLC; a shift towards a more apolar elution position of the derivatized product should be found as compared to the normal peptide, Fig. 3b.

*Deprotection of derivatized peptides*

The t-BOC group was removed by treatment with concentrated acid, undiluted formic or trifluoroacetic acid. After this treatment the peptide was purified by HPLC as shown in Fig. 4. The deprotected, *i.e.*, tyrosylated peptides are eluted slightly later than the original peptide due to the extra apolar amino acid, tyrosine. After deprotection, the new tyrosyl residue at the amino terminus of the peptides can be verified by amino acid sequence determination, as demonstrated in Table I for PP and the 1-12-fragment of somatostatin-28. The deprotection, particularly with formic acid, can produce heterogeneity of the peptide; in the case of PP a minor fraction of the deprotected peptide is eluted as a separate peak (Fig. 4).

*Iodination of derivatized peptides*

We have chosen in most instances to iodinate the derivatized peptide directly

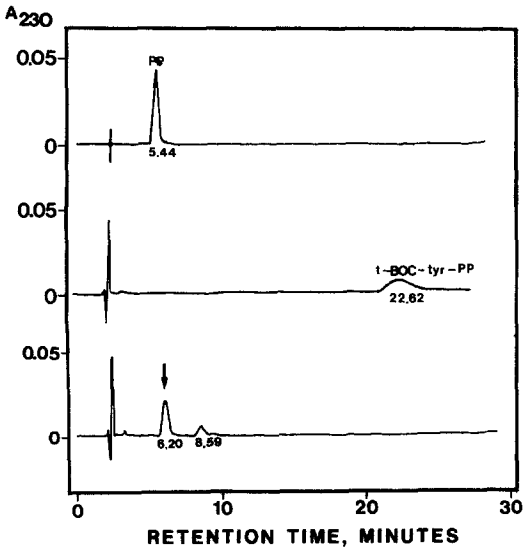


Fig. 4. Deprotection of derivatized, pancreatic polypeptide. Isocratic HPLC analysis of underivatized pancreatic polypeptide (PP), derivatized peptide, t-BOC-Tyr-PP, and the deprotected peptide (lower). The solid arrow indicates the peptide identified as $Tyr_0$-PP by amino acid sequence analysis, see Table I. The column used in Fig. 1 was eluted with 35% acetonitrile in 0.1% TFA–water.

TABLE I

DETERMINATION OF THE AMINO-TERMINAL AMINO ACID SEQUENCE IN DERIVATIZED, DEBLOCKED PEPTIDES PURIFIED BY HPLC

The amino-terminal sequence of natural, bovine PP (BPP) is Ala-Pro-Leu-Glu-Pro-Glu-Tyr- and the amino-terminal sequence of fragment-1-12 of somatostatin-28 is Ser-Ala-Asn-Ser-Asn-Pro-Ala-.

| *Sequence cycle* | *BPP** | | *Somatostatin-28* | |
|---|---|---|---|---|
| | *Amino acid* | *Yield (pmol)*** | *Amino acid* | *Yield (pmol)*** |
| 1 | Tyr | 79 | Tyr | 248 |
| 2 | Ala | 65 | Ser | 44 |
| 3 | Pro | 67 | Ala | 286 |
| 4 | Leu | 58 | Asn | 158 |
| 5 | Glu | 31 | Ser | 26 |
| 6 | Pro | 47 | Asn | 155 |
| 7 | Glu | 25 | Pro | 170 |
| 8 | Tyr | 43 | Ala | 223 |

* $Tyr_0$-BPP collected as the peak eluted after 6.20 min in Fig. 4, lower panel.

** Yield of the phenylthiohydantoin derivative of the amino acid in each cycle. Around 200 pmol BPP and 750 pmol somatostatin-28 were applied to the glass filter.

without removal of the protecting t-BOC group. The iodination was performed with the insoluble chloroglycouril as the oxidative agent (Serva), as this causes minimum oxidative damage to larger peptides. The purification of the iodinated, derivatized pancreatic eicosapeptide by isocratic elution in HPLC is shown in Fig. 5a. The peptides are radioiodinated in the "new" amino-terminal tyrosyl residue and therefore react well with antisera which by specific coupling are usually directed towards the carboxy-terminal end of the molecule. In the present case the antisera against the pancreatic eicosapeptide were developed against the peptide coupled to bovine serum albumin (BSA) in its carboxy-terminal α-amino group using difluorodinitrobenzene[10], Fig. 5b and c.

One of the advantages of the original conjugation methods was that the peptide was not in contact with strong oxidants, since the derivatizing reagent was iodinated and purified before it was added to the peptide[6,7,9]. Two different methodological developments within recent years have to some extent made this protection of the peptide superfluous. First, by using the sparingly soluble chloroglycouryl as the oxidative agent, only minimum oxidative damage to peptides occurs[5]. Secondly, because oxidation of methionine residues produces a considerable increase in polarity of the peptide, the purification of the radiolabelled peptides on isocratically eluted HPLC systems will separate oxidized peptides, if at all present, from the non-oxidized forms.

We have chosen to use the Assoian reagent for the introduction of a chemical moiety which can accept radioiodine by substitution. The Bolton–Hunter reagent has previously been used in this way[15–17]. Both reagents use the same active group, N-hydroxysuccinimide ester, for coupling to amino groups on the peptide. Conjugation and subsequent radiolabelling at the amino terminus of the peptides is

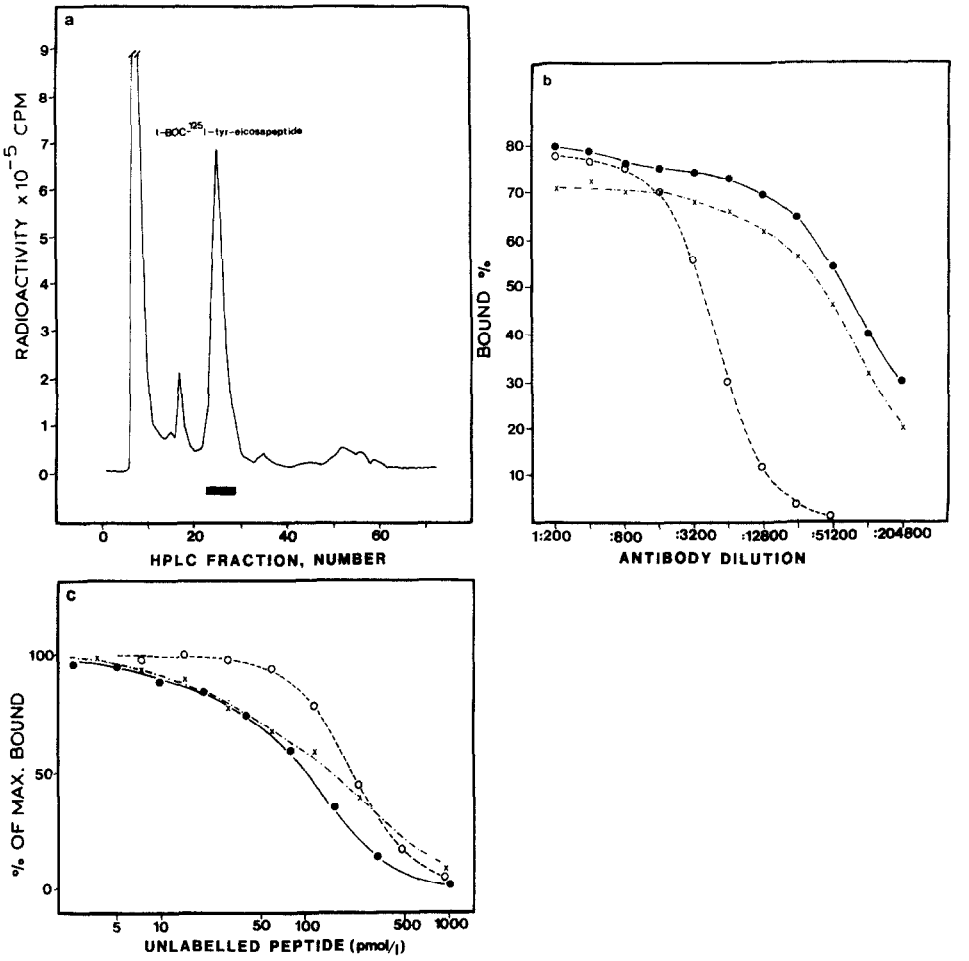


Fig. 5. Radioiodination and radioimmunoassay of derivatized pancreatic eicosapeptide. (a) HPLC purification of t-BOC-Tyr-derivatized pancreatic eicosapeptide radioiodinated by oxidation with 1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril. A column similar to the one used in Fig. 1 was eluted isocratically with 28% (v/v) acetonitrile in 0.1% (v/v) TFA in water. The non-iodinated peptide derivative was eluted in fraction 18. The solid bar indicates the fractions used in antibody-binding studies. (b) Binding of radioiodinated, derivatized eicosapeptide to three different antisera, 3201-1 (×), 3202-2 (●) and 3204-L (○). (c) Displacement of natural pancreatic eicosapeptide of radiolabelled, derivatized eicosapeptide binding to different antisera: antiserum 3201-1 diluted 1:6 × $10^4$ (×); antiserum 3202-2 diluted 1:1 × $10^5$ (●) and antiserum 3204-L diluted 1:2.5 × $10^3$ (○).

advantageous since most antisera are raised against peptides coupled to carrier proteins by methods which also use the amino functions of the peptides, *e.g.*, carbodiimide or difluorodinitrobenzene. Hence, it may be advantageous that both the coupling method and the conjugative radiolabelling method leave the carboxy-terminal end of the peptide intact. The Assoian reagent has an extra advantage, as it offers the possibility to regenerate the correct amino group at the beginning of the peptide by removal of the protecting t-BOC group as demonstrated in Fig. 2 and Table

I. This may be important, if the radioiodinated peptide is to be used in cross-linking experiments in receptor studies[18].

ACKNOWLEDGEMENTS

U.G. and H.V.N. received pregraduate scholarship from the Weimann Foundation during this work. T.W.S. is the recipient of a Professorship in Molecular Endocrinology from the Danish Medical Research Council and the Weimann Foundation. The laboratory is also supported by grants from the Danish Natural Science Research Foundation, the Carlsberg Foundation and the NOVO Foundation. Dr. Mairead O'Hare is thanked for critical reading and Henny Jensen is thanked for secretarial help during the preparation of the manuscript.

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