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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS

LVII*. ANALYSIS OF RADIOIODINATED THYROTROPIN POLYPEPTIDES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The chromatographic behaviour of bovine and human thyrotropin, radiolabelled stoichiometrically with lactoperoxidase, on octadecylsilicas and other reversed-phase *n*-alkylsilicas has been investigated. As part of this investigation the effects of a variety of elution systems on resolution and recovery have been examined. Analysis of the tryptic peptides of radioiodinated bovine thyrotropin (bTSH) preparations by reversed-phase high-performance liquid chromatographic mapping methods resulted in the separation of more than fourteen major radioactive peptide components. The data indicate that bTSH is radioiodinated with unequal incorporation into both the α- and the β-subunits. Further, assessment of the level of microheterogeneity of radiolabelled bTSH preparations can be achieved with these reversed-phase techniques.

INTRODUCTION

Thyrotropin (TSH) is a pituitary glycoprotein hormone, composed of non-covalently associated α- and β-subunits, which interact with specific receptors on thyroid cells to stimulate *inter alia* thyroid hormone synthesis and secretion. In the past, the purification of TSH to homogeneity from human, bovine, ovine, whale and rat pituitaries has presented a variety of technical difficulties[1–6]. With conventional isolation methods, such as ion-exchange chromatography, lectin-affinity chromatography, and gel-permeation chromatography, TSH preparation are invariably obtained which exhibit multiple bands of bioactivity on polyacrylamide gel electrophoresis and isoelectric focusing. Carbohydrate heterogeneity in the glycosylated subunits may be responsible for part of this behaviour. However, intracellular post-translational processing and artefactual degradation during isolation may also play significant roles in the generation of microheterogeneous forms of this protein. With increasing use of radioiodinated TSH preparations for radioimmuno- and receptor-

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assays, it is clearly desirable that the radiotracer should be as similar to the native hormone in biological and physicochemical properties as possible. With the current generation of high-performance liquid chromatographic (HPLC) protocols, based in particular around ion-exchange and reversed-phase procedures, rapid, high-resolution separations of radioinert and radiolabelled polypeptide hormones are now attainable. In previous studies[7–11] from this laboratory the chromatographic separation of a variety of pituitary proteins and polypeptide hormones on monodisperse anion-exchange supports and small- or large-pore alkylsilicas have been described. The present study documents the chromatographic behaviour of radiolabelled bovine and human TSH and their derived subunits in gradient elution reversed-phase HPLC on small- and large-pore, microparticulate *n*-alkylsilicas.

EXPERIMENTAL

Materials

Highly purified bovine thyrotropin (bTSH) (30–40 I.U./mg) and the isolated α-subunit and the isolated β-subunit of bTSH (designated bTSH-αA and bTSH-βA, respectively) were kindly provided by Dr. J. G. Pierce, (UCLA, CA, U.S.A.). Purified human thyrotrophin (hTSH) was isolated in this laboratory from pituitary fractions provided by the Human Pituitary Advisory Committee, Department of Health (Australia). Bovine lactoperoxidase (with E_{max} 420 nm/280 nm 0.70) was obtained from Calbiochem-Behring (Australia), and sodium [^{125}I]iodide ($Na^{125}I$; carrier-free) was purchased from The Radiochemical Centre (Amersham, U.K.). Sephadex G-100 (Superfine) was obtained from Pharmacia (Uppsala, Sweden). Acetonitrile (HPLC grade) was purchased from Waters Assoc. (Milford, MA, U.S.A.). Distilled water was deionized using a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Ammonium hydrogen carbonate (NH_4HCO_3) was obtained from Ajax Chemicals (Sydney, Australia). All other chemicals were of AnalaR reagent grade. Human serum albumin (HSA) was obtained from Commonwealth Serum Laboratories, (Parkville, Australia).

Equipment

HPLC separations were carried our with a Waters Assoc. HPLC system which consisted of two M6000A solvent delivery units, a U6K universal injector and a M660 solvent programmer. The μBondapak C_{18} and alkylphenyl columns (30 cm $\times$ 0.4 cm I.D.; d_p = 10 μm) were purchased from Waters Assoc., whilst the Bakerbond C_{18} columns (25 cm $\times$ 0.46 cm I.D.; d_p = 5 μm) were generously donated by J. T. Baker (Phillipsburg, NJ, U.S.A.). The preparation and characteristics of the Merck LiChrospher, bonded with a trimethylsilyl stationary phase, have been described elsewhere[12,13]. Radioactivity was quantitated with a Packard Auto Gamma Spectrometer, Model 5260 MD, U.S.A.).

Methods

All proteins were iodinated with ^{125}I by a modified lactoperoxidase procedure[14] based on the method described by Lissitzky *et al.*[15]. Free ^{125}I was removed by chromatography on a Sephadex G-100 (Superfine) (30 cm $\times$ 1.2 cm I.D.) column, equilibrated in 20 m*M* Tris–HCl, 1 m*M* EDTA, 0.5 mg/ml HSA, pH 7.40. The ra-

dioiodinated tracers were stored at 4°C. Specific activities achieved were 30–60 μCi/μg (bTSH or hTSH) and 80–120 μCi/μg (bTSH-αA, bTSH-βA).

Radioiodinated subunits of bTSH were dissociated from iodinated intact bTSH by incubation in 1 *M* propionic acid[16] (final concentration) for *ca.* 18 h at room temperature and aliquots (100–500 μl) were subsequently lyophilised. The subunits (designated ^{125}I-bTSH-αD and ^{125}I-bTSH-βD) were separated by chromatography on a Sephadex G-100 (Superfine) column (95 cm × 1.5 cm; flow-rate 5 ml/h), equilibrated in 50 m*M* NH_4HCO_3–0.05% HSA (w/v).

Samples of radiolabelled polypeptides and proteins were chromatographed at ambient temperature by reversed-phase HPLC. Typically, 10-μl samples were loaded onto a μBondapak C_{18} column, pre-equilibrated in 100 m*M* NH_4HCO_3 at a flow-rate of 1 ml/min. A 120-min linear gradient to 100 m*M* NH_4HCO_3, 50% acetonitrile was started immediately upon injection and 1-min fractions were collected for γ-counting and subsequent analysis. Various other elution conditions were employed with the Bakerbond C_{18} columns, and the other reversed-phase supports.

RESULTS AND DISCUSSION

Radioiodinated TSHs are now widely used as ligands for the detection by radioimmunoassay procedures of circulating levels of TSH in plasma and in studies on the interaction between TSH and its plasma membrane receptors. Generally, the radioiodination of TSH is carried out by the stoichiometric chloramine-T or the lactoperoxidase procedures. Typically, the reaction samples are desalted subsequently on Sephadex G-100 Superfine in order to remove free ^{125}I, but no further chromatographic purification of the site-specific radiolabelled protein hormone components is attempted. In view of recent experiences[17–19] on the separation of site-specific radioiodinated insulins, growth hormone, and other polypeptide hormones by reversed-phase HPLC procudures, similar chromatographic stategies were attempted with radioiodinated bovine and human TSH. Fig. 1a shows the gradient elution profile of a ^{125}I-bTSH preparation, chromatographed on a μBondapak C_{18} column with a primary 100 m*M* ammonium bicarbonate buffer and a 0–50% acetonitrile gradient. With this elution system, more than 90% of the radioactivity applied to the column was recovered with little evidence of dissociation of the radiolabelled protein to its corresponding radiolabelled α- or β-subunits. Comparable results were obtained with radioiodinated human TSH (^{125}I-hTSH) under similar elution conditions. When different stationary phase and mobile phase systems were chosen to permit elution under gradient conditions of the ^{125}I-bTSH and ^{125}I-hTSH glycoproteins at pH values above pH 3.0, recoveries ranged between 85 and 95% (Table I). In Figs. 1b and 1c are shown the elution profiles for bTSH-αA and bTSH-βA, independently radioiodinated by the lactoperoxidase procedure[14,15].

Because of the hydrophobic nature of the iodo group, radioiodinated polypeptides generally exhibit slightly larger elution volumes than the corresponding radioinert polypeptides on microparticulate *n*-alkylsilicas. Furthermore, bandwidths of radiolabelled and radioinert polypeptides are usually comparable for molecules smaller than 40,000 daltons. In fact, narrow elution zones are generally observed[10] for polypeptides under gradient conditions similar to those used in the present study. Despite the high recovery of ^{125}I-TSHs that could be obtained under the above

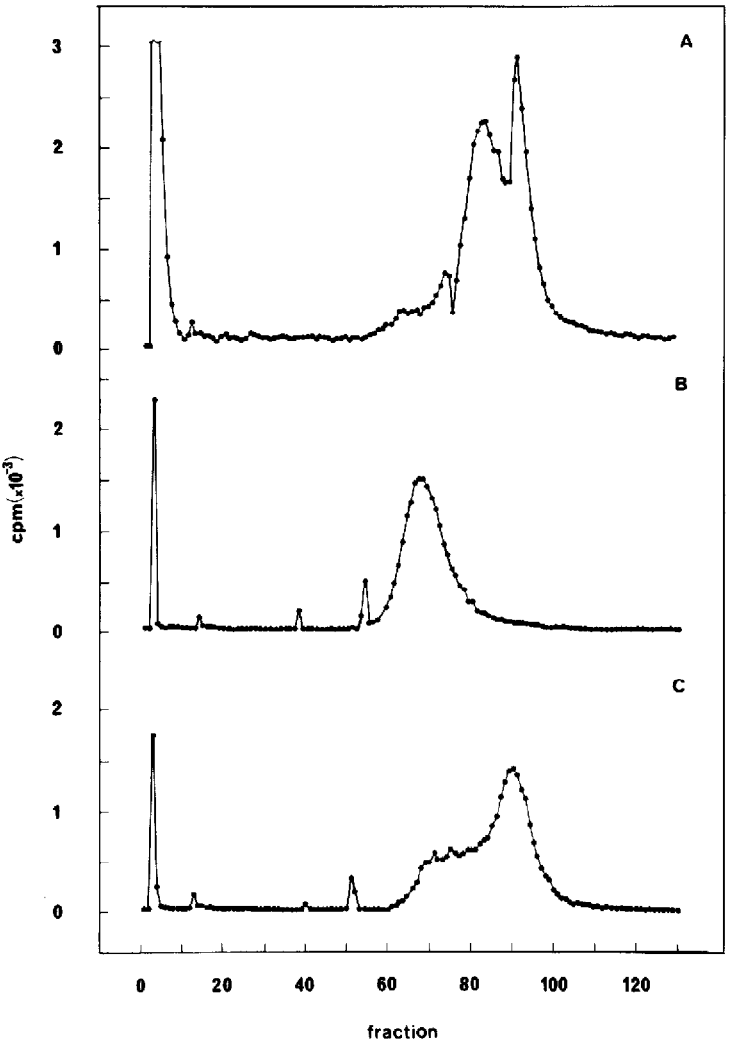

Fig. 1. Elution profiles of bTSH (A), bTSH-α(B) and bTSH-β(C), independently radioiodinated by the lactoperoxidase procedure, chromatographed on a μBondapak C_{18} column with a 120-min linear gradient from 100 m*M* ammonium bicarbonate to acetonitrile–water (50:50)–100 m*M* ammonium bicarbonate at a flow-rate of 1.0 ml/min.

reversed-phase HPLC conditions, the peak shape and symmetry appear anomalous when compared with our earlier experiences with other radioiodinated polypeptides. However, radiolabelled luteotropin (LH), human chorionic gonadotropin (hCG), and follitropin (FSH), three structurally related glycoprotein hormones, which are also composed of associated α- and β-subunits, exhibit[19,20] similar chromatographic behaviour on the μBondapak C_{18} stationary phase as well as on other small-pore *n*-alkylsilicas. Peak asymmetry for polypeptides eluted from *n*-alkylsilicas under isocratic and gradient conditions has previously been associated with stationary phases

TABLE I

RECOVERIES OF ^{125}I-LABELLED TSH AND RELATED SUBUNITS UNDER DIFFERENT CHROMATOGRAPHIC CONDITIONS

Sample	*Column*	*Elution condition**	*Recovery*
^{125}I-bTSH	μBondapak C_{18}	1	103
^{125}I-bTSH-α	μBondapak C_{18}	1	75
^{125}I-bTSH-β	μBondapak C^{18}	1	61
^{125}I-hTSH	μBondapak C_{18}	2	82
^{125}I-hTSH	μBondapak alkylphenyl	3	94
^{125}I-hTSH	μBondapak alkylphenyl	4	95
^{125}I-hTSH	LiChrospher C_1	2	85
^{125}I-bTSH	Bakerbond C_{18}	1	92
^{125}I-bTSH-α	Bakerbond C_{18}	1	92
^{125}I-bTSH-β	Bakerbond C_{18}	1	92

* Elution condition 1: linear 120-min gradient from 100 m*M* ammonium bicarbonate (A) to 50% acetonitrile in A (v/v) at a flow-rate of 1.0 ml/min. Elution condition 2: linear 60-min gradient from 200 m*M* ammonium bicarbonate (A) to 50% acetonitrile in A (v/v) at a flow-rate of 2.0 ml/min. Elution condition 3: linear 60-min gradient from 200 m*M* ammonium acetate, pH 7.0, to acetonitrile–water (60:40)–80 m*M* acetic acid, pH 3.5 at a flow-rate of 2.0 ml/min. Elution condition 4: linear 60-min gradient from 0 to 70% B, followed by a step to 100%, where the initial solvent (A) was 100 m*M* ammonium acetate, pH 7.0, and B was acetonitrile–water (60:40)–30 m*M* acetic acid 70 m*M* trifluoroacetic acid, pH 2.0, at a flow-rate of 2.0 ml/min.

of small porosities, inappropriate *n*-alkyl chain ligand densities or incomplete stationary-phase endcap coverage[21,22]. However, as is evident from Fig. 2, the use of a large-pore fully endcapped octadecylsilica with a nominal porosity of 33 nm did not result in any significant improvement in peak shape for ^{125}I-bTSH or the dissociated radiolabelled α- and β-subunits (^{125}I-bTSH-αD and ^{125}I-bTSH-βD, respectively) derived from 1 *M* propionic acid treatment[14] of ^{125}I-bTSH.

Several possibilities immediately suggest themselves to account for the observed chromatographic behaviour. For example, current preparations of bovine TSH, including that used in the present investigation, exhibit isoform microheterogeneity. Although the broadened peaks observed for radioiodinated TSH preparations may be a consequence of unfavourable kinetic processes, associated with subunit–subunit or subunit–stationary phase interactions, a more likely explanation involves the peak envelope containing incompletely resolved bTSH of different iodination state, oxidation state or structural and microheterogeneity. Previously, we have observed[7] that separation of radiolabelled preparations of bTSH by high-performance ion-exchange chromatography is also associated with diffuse peak profiles. The second possibility is thus favoured in view of the similarity of the elution features obtained by two procedures of alternative chromatographic selectivity. Zone broadening and peak asymmetry for proteins separated on *n*-alkylsilicas are known[22] to be due to the participation of slow, secondary equilibrium processes with some protein solutes, low diffusibility, and unfavourable mass transfer from the stationary phase. However, these effects are usually associated in gradient elution reversed-phase HPLC of proteins with low recoveries and 'ghost' peaks on subsequent repetition of gradient elution[10]. This 'ghost' peak behaviour was not observed with the elution

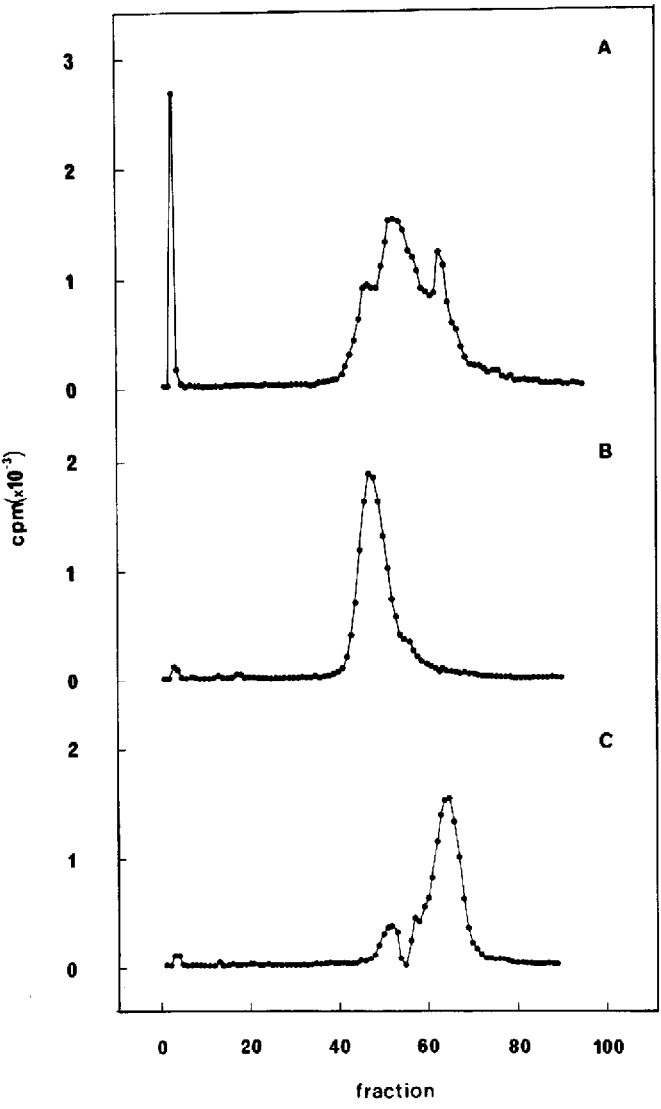

Fig. 2. Elution profiles for ^{125}I-bTSH (A), as well as ^{125}I-bTSH-α(B) and ^{125}I-bTSH-β(C), prepared by the propionic acid dissociation procedure, chromatographed on a Bakerbond C_{18} column with a 90-min linear gradient. The other elution conditions are the same as given in the legend to Fig. 1.

system used in the present study, where high recoveries were routinely obtained with all radiolabelled TSH polypeptides. More importantly, rechromatography under identical conditions of individual fractions recovered from these reversed-phase HPLC separations confirmed that the band dispersion was not induced by the chromatographic conditions, *i.e.* well defined peaks of good plate number were obtained with no change in retention behaviour. In addition, separation of the tryptic peptides of reduced and alkylated ^{125}I-bTSH by reversed-phase HPLC revealed fourteen major peaks of radioactivity (Fig. 3). Since on complete digestion with pronase these

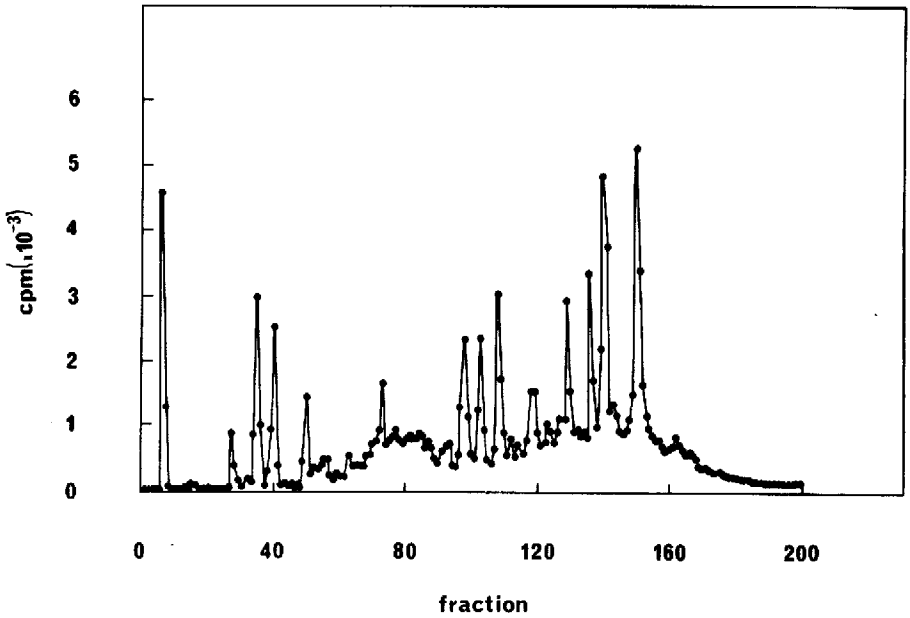

Fig. 3. Separation of the tryptic peptides of reduced and carboxymethylated ^{125}I-bTSH on a Bakerbond C_{18} column with a 100-min linear gradient from 100 m*M* ammonium bicarbonate to acetonitrile–water (30:70)–100 m*M* ammonium bicarbonate at a flow-rate of 1.0 ml/min. Collected fractions (500 μl) were counted for radioactivity, the total recovery being greater than 93%.

^{125}I-bTSH preparations yield[14] predominantly radiolabelled 3-iodotyrosine with only trace amounts of the total protein-bound ^{125}I as 3,5-di-iodotyrosine, the major radioactive tryptic peptides presumably are derived from microheterogeneous bTSH species, either singly labelled to similar specific activity at the same tyrosine residue or alternatively labelled to variable specific activities at different tyrosine residues on the TSH surface. The available evidence supports the latter possibility. In addition, polymorphism of the ^{125}I-bTSH preparation due to oxidative modification, deamidation, etc. would also account for the minor radioactive tryptic peptides apparent in the chromatographic separation shown in Fig. 3. It has been reported[23–25] that radioiodination by the chloramine T or lactoperoxidase methods of ovine and porcine LH as well as hCG occurs almost exclusively in the α-subunit. The present data, as well as other studies[14], demonstrate that intact TSH on radioiodination incorporates iodine into both subunits. A singly labelled, site-specific ^{125}I-bTSH would have a specific activity of 67 μCi/μg[26], based on a molecular weight for this protein of 28,000 and an average biological activity of 30 I.U./mg. The specific activity of the preparations of ^{125}I-bTSH used in the present study was between 30 and 60 μCi/μg. If one iodine atom per tyrosine residue was incorporated into each bTSH molecule on a mole average, then on the basis of known amino acid sequence[27] there would be sixteen theoretically possible monoiodinated TSH derivatives, sixteen possible monoiodinated tyrosine containing tryptic peptides and a correspondingly larger number of di-, tri-, etc., iodinated species. Since incorporation would be more favoured into surface-accessible tyrosine residues and not into internal residues or

those associated with subunit contact regions[28], non-uniform incorporation into these tyrosine residues is anticipated. The elution profile for radioactivity for the tryptic peptides of ^{125}I-bTSH and its radiolabelled α- and β-subunits is consistent with this incorporation pattern. Furthermore, evidence from biological activity, binding activity and electrophoretic mobility measurements of radiolabelled TSH indicates[14,29–31] that the broad profile seen for ^{125}I-TSH on hydrophobic *n*-alylsilicas and hydrophilic silicas, such as the glycidoxypropylsilicas, is consistent with a heterogeneous protein distribution containing TSH species, iodinated at different sites and exhibiting structural variability due to oxidation of sensitive residues in the TSH amino acid sequence, *e.g.* at methionine residues, as well as due to the method of isolation of the TSH itself. The fact that in some HPLC chromatographic systems the majority of the radioactivity and the bioactivity for ^{125}I-labelled TSH species are not coincident[28,29] suggests that several iodination procedures currently in use preferentially modify crucial tyrosine residues associated with biological function without necessarily impairing membrane receptor binding activity. In associated studies[5,14], detailed evaluation of the receptor binding and electrophoretic characteristics of radioiodinated bTSH and oTSHs, purified by these different chromatographic procedures are presented.

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