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High-performance liquid chromatography of rat and mouse islet polypeptides: potential risk of oxidation of methionine residues during sample preparation[a]

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ABSTRACT

After preparative high-performance liquid chromatography of mouse islet culture medium, concentrated on disposable C_{18} cartridges (Sep-Pak), an unexpected insulin immunoreactive peak eluting earlier than mouse insulin I and II was detected. Molecular mass determination by mass spectrometry supported its suspected identity as methionine sulphoxide insulin II. We have examined the formation of Met-O derivatives of insulin II, glucagon and pancreatic polypeptide during sample preparation (Sep-Pak and Speed-Vac concentrating). The oxidation of methionine residues was found to depend very much on the buffer, the organic modifier and the procedure. In particular the use of methanol–trifluoroacetic acid resulted in extensive oxidation. The oxidation could be minimized by adding 2 m*M* dithiothreitol to the buffer and by degassing and/or nitrogen-bubbling of the buffer. Minimal formation of Met-O derivatives is important for the quantitation of methionine-containing polypeptides.

INTRODUCTION

Studies of the biosynthesis of proinsulins and their conversion into insulins and C-peptides in the rat and mouse endocrine pancreas depend on accessible reversed-phase high-performance liquid chromatographic (RP-HPLC) analyses capable of separating all the polypeptides involved.

We have recently described two HPLC systems that can separate the two non-allelic insulins (I and II) from rat and mouse, as well as the two C-peptides (I and II), and the two proinsulins (I and II) [1]. In order to identify the individual mouse polypeptides by amino acid analysis and micro-sequencing [1] we concentrated medium from cultured mouse islets on disposable C_{18} cartridges (Sep-Pak). In some cases this procedure resulted in an unexpected peak which reacted as insulin by radioimmunassay after RP-HPLC separation of the concentrated medium. Since insulin II, in contrast to insulin I, contains a methionine residue in

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position 29 of the B-chain, oxidation to methionine sulphoxide in insulin II might have happened during sample preparation [2–5].

The aim of the present study was to investigate the influence of buffer components, organic modifiers and isolation procedures on the formation of Met-O derivatives of insulin II, as well as of glucagon and pancreatic polypeptide, in order to minimize the oxidation of the methionine residues.

EXPERIMENTAL

Reagents

Trifluoroacetic acid (TFA, Peptide Synthesis grade) was from Applied Biosystems and acetonitrile (HPLC grade S) was from Rathburn. All other chemicals were of analytical-reagent grade. Distilled water was purified on a Millipore Milli-Q plant, and all buffers were filtered (0.45 μm, Millipore) and degassed by vacuum/ultrasound before use.

Standards

As a source of rat insulin I and II and C-peptide I and II, medium from newborn rat islet cells cultured in RPMI 1640 supplemented with 2% human serum in the presence of 1 μg/ml human growth hormone was used. The medium contained 44 μg/ml of insulin I and II and equimolar amounts of C-peptide I and II. Rat pancreatic polypeptide was obtained from Penninsula and porcine glucagon from Sigma. Rat insulin II was prepared from the culture medium using preparative HPLC.

Chromatography

The HPLC system consisted of Waters M6000 A pumps, a WISP 710A, a 660 solvent programmer, a 730 data module and a Pye Unicam LC-UV detector. The column was LiChrosorb RP-18, 5 μm particle size, 250 x 4.0 mm I.D. (Merck), eluted at 1.0 ml/min with a linear gradient of acetonitrile (30 to 36%) in 0.1% TFA during 60 min. The column eluate was monitored at 210 nm, peaks were collected either manually or by collecting 0.5-min fractions in a FRAC 300 fraction collector (Pharmacia). All separations were carried out at room temperature.

Sample preparation using Sep-Pak

Disposable C_{18} cartridges (Sep-Pak, Waters) were used. The organic eluent (B) contained 90% (v/v) of the organic modifier, which was acetonitrile, 2-propanol or methanol. The aqueous eluent (A) was water, 1 *M* acetic acid or 0.1% TFA. The Sep-Pak cartridge was flushed with 10 ml of B and 10 ml of A, and the sample was applied, followed by washing with 10 ml of A and elution with 1.5 ml of B into an Eppendorf tube. The sample was concentrated in a Speed-Vac concentrator (Savant).

Radioimmunoassay (RIA)

Collected fractions containing TFA–acetonitrile were dried in a Speed-Vac concentrator, and radioimmunological determination of insulin was carried out using rat insulin (Novo) as a standard and anti-mouse insulin antibodies (developed in this laboratory) as previously described [6].

Hydrogen peroxide oxidation

A 1-ml volume of a solution containing 1% hydrogen peroxide (v/v) in 3 *M* acetic acid was evaporated in the Speed-Vac concentrator in a separate tube, together with 2–40 μg samples of glucagon, pancreatic polypeptide or insulin II dissolved in 3 *M* acetic acid, as well as rat culture medium diluted 1:1 (v/v) with 3 *M* acetic acid.

Determination of molecular mass

The molecular masses (M_r) of the polypeptides and their oxidized forms isolated after HPLC were determined by mass spectrometry (MS). The mass spectra were obtained on a Bio-Ion 20 plasma desorption mass spectrometer [7]. Prior to analysis the samples were applied to nitrocellulose targets prepared on Mylar foil. Spectra were accumulated for $5 \cdot 10^6$ fission events.

RESULTS AND DISCUSSION

Isolation and identification of polypeptides and proteins present in very small amounts in complex solutions, such as cell culture media, normally require a concentration step before the HPLC analysis, and especially for large volumes it is convenient to use disposable Sep-Pak C_{18} cartridges for sample preparation.

Mouse islet culture media (100 ml) were loaded on a Sep-Pak, the cartridge was washed with 10 ml of 30% methanol (thereby eluting several non-peptide culture medium constituents) and thereafter the polypeptides were eluted with 90% methanol. Fig. 1 shows the HPLC analysis of the concentrated medium. Although several sample components with no relevance to the C-peptides and insulins were removed before HPLC, the presence of numerous UV-absorbing components made a direct localization of the islet polypeptides difficult: mouse C-peptide I and II, expected to elute at 25–30 min, could not be distinguished from neighbouring components, and only after insulin RIA were the insulins localized in three peaks. Peaks 2 and 3 eluted at the same retention times as rat insulin I and II, respectively (rat and mouse insulins are identical).

In order to verify the hypothesis that insulin peak 1 could be methionine sulphoxide insulin II, rat islet culture medium was oxidized with hydrogen peroxide reported to oxidize methionine to methionine sulphoxide [8] as described above. HPLC analysis of this sample showed that an additional component with a lower retention time (marked with an arrow, Fig. 2) was formed during oxidation simultaneously with a decrease of the amount of insulin II. The lower

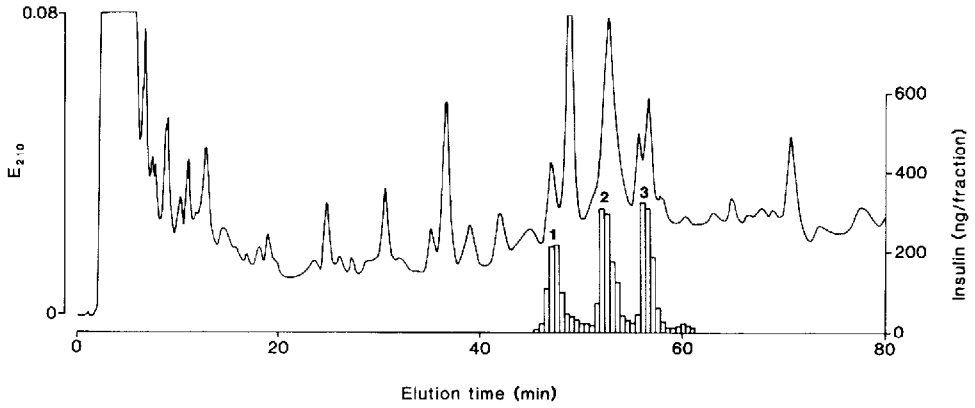

Fig. 1. HPLC separation of 40 ml of Sep-Pak-concentrated mouse islet culture medium using a LiChrosorb RP-18 column (250 x 4.0 mm I.D.) eluted at a flow-rate of 1.0 ml/min with a concave acetonitrile gradient (gradient 7, 30 to 36%) in 0.1% TFA during 60 min; 0.5-min fractions were collected and dried in a Speed-Vac, and the insulin content was determined using RIA (histogram).

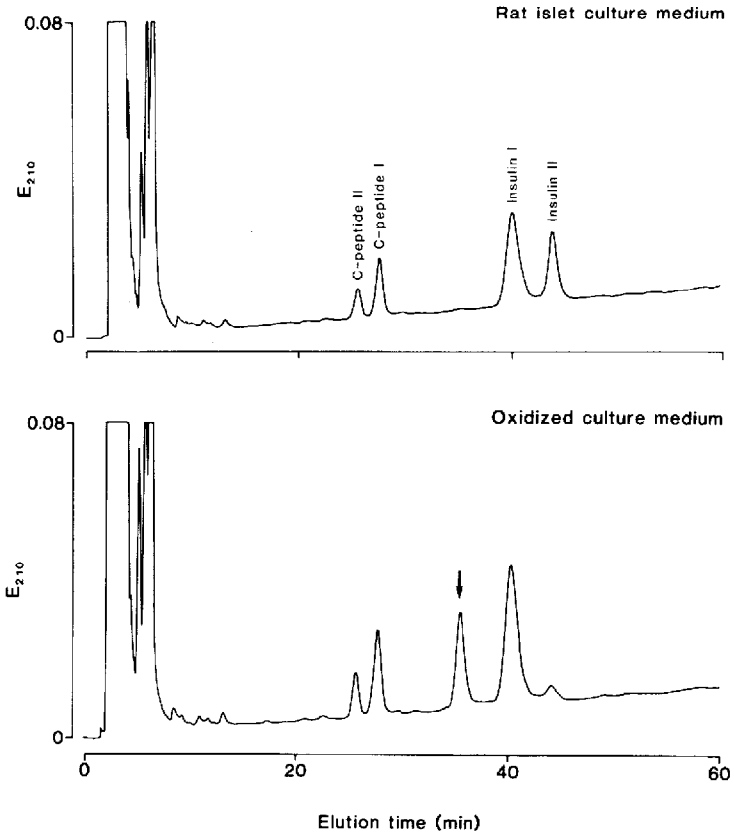

Fig. 2. HPLC separation of 50 μl of rat islet cell culture medium and 75 μl of H_2O_2-oxidized medium using a LiChrosorb RP-18 column (250 x 4.0 mm I.D.) eluted at a flow-rate of 1.0 ml/min with a linear aceto-

retention time was expected since the Met-O derivatives are reported to have higher polarity [10]. The retention time of this newly formed component relative to that of insulin II could not directly be compared with that of peak 1 in Fig. 1, since different acetonitrile gradients were used (see legends to Figs. 1 and 2). The identification of rat insulin I and II (Fig. 2, upper panel) was based on amino acid analysis and sequencing, as well as by RIA as previously described [1].

Likewise, oxidation of other methionine-containing islet polypeptides resulted in additional peaks with lower retention times as shown for glucagon (Fig. 3, two additional peaks) and pancreatic polypeptide (Fig. 4). The additional peaks (marked with arrows in Figs. 2–4) as well as the authentic polypeptides were collected after HPLC and subjected to MS. If necessary, the samples were concentrated in the Speed-Vac concentrator before MS. The resulting molecular masses are shown in Table I, together with the theoretically calculated values. In the case of glucagon, two additional peaks were detected (Fig. 3) that had the same molecular mass (Table I), although only one methionine residue is present. Two different methionine sulphoxide forms exist, the D- and L-forms, but it is

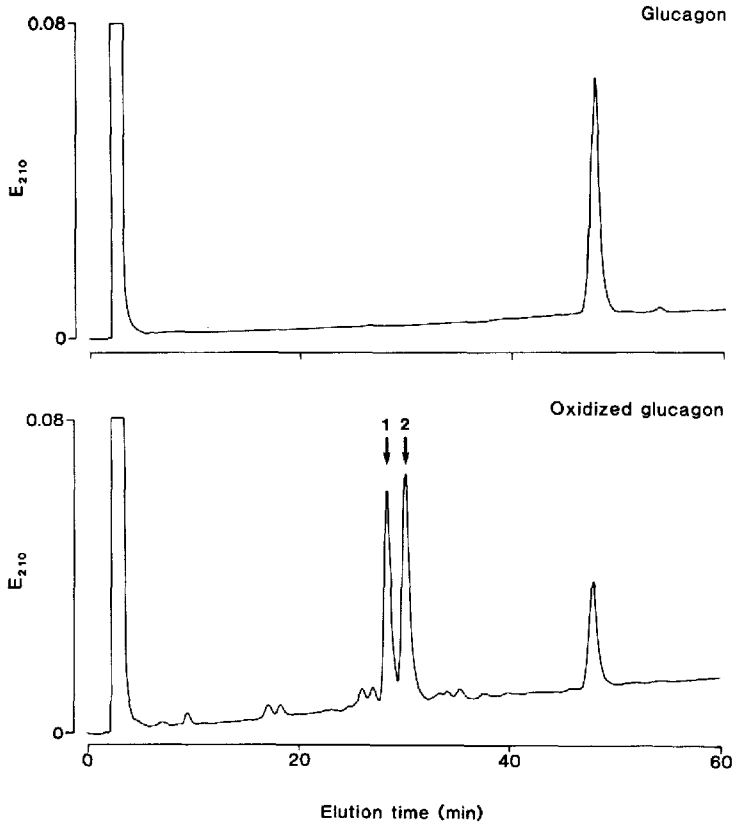

Fig. 3. HPLC separation of glucagon (2 μg) and H_2O_2-oxidized glucagon (3 μg). Conditions as in Fig. 2.

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