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PURIFICATION OF PORCINE PROINSULIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A procedure has been developed for purification of porcine proinsulin by high-performance liquid chromatography from a preparation obtained as a side product during the Sephadex G-50 gel filtration of an impure porcine insulin preparation. Reversed-phase chromatography was carried out on octadecylsilica as the stationary phase with graded mixtures of acetonitrile or methanol–acetonitrile and phosphate buffer pH 2.4 as the mobile phase. The crude preparation separated into five different groups of proteins, the proinsulin-containing peak being identified by the co-eluting internal proinsulin marker. After purification by conventional procedures (separation, pooling, freeze drying, desalting, reprecipitation and drying) this peak fraction was rechromatographed by high-performance liquid chromatography (for final purification) to give a single peak protein which had identical electrophoretic mobility to that of commercial porcine proinsulin, and which converted to a protein with electrophoretic mobility similar to that of porcine insulin.

INTRODUCTION

Proinsulin is the intermediate or second stage biosynthetic precursor of insulin and is probably of significance for the transport of the newly synthesised protein and its subsequent storage in the β-granules of the pancreatic B-cells[1]. Compared with insulin, proinsulin has an additional short chain peptide, called the connecting or the C-peptide, joining the otherwise free amino and carboxyl ends of the insulin A and B chains, respectively. The amino acid sequence of the C-peptide is highly species-specific, unlike that of the insulin molecule which shows, with few exceptions, a high degree of structural similarity throughout the species so far studied[1,2].

Proinsulin is a very useful antigen for raising species-specific antibodies binding C-peptide since the latter is poorly antigenic[1,3]. Highly purified hormones are necessary both for preparing specific antibodies for immunochemical assays, and also for therapeutic use[1,2]. Purification of proinsulin by ion-exchange chromatography has been described[4,5], and more recently there have been preliminary reports on the analysis by high-performance liquid chromatography (HPLC) of crystalline peptide

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hormones commercially purified for therapeutic use[6–8]. The present work aimed to purify proinsulin by preparative HPLC as an alternative to conventional ion-exchange chromatography which we found[9] to be inefficient in the purification of insulin itself.

MATERIALS

Solvents were of HPLC grade (Rathburn Chemicals, Walkerburn, Great Britain). Crude porcine insulin, crystallised twice (Lot No. S 2902181), containing approximately 3% "b" component, was a generous gift from Dr. Frank Petersen of Novo Research Institute (Bagsvaerd, Denmark). Purified porcine proinsulin and the therapeutic porcine insulins Actrapid and Monotard were purchased from the Novo Research Institute.

Chymotrypsin-free trypsin type XI was purchased from Sigma London Chemical Company (Poole, Great Britain).

METHODS

Gel chromatography

Batches of crude insulin (250–300 mg) were applied to a 5 × 80 cm column of Sephadex G-50 fine (Pharmacia, Uppsala, Sweden) equilibrated and run with 1.0 mol/l acetic acid at 16 ml/h. The "b" component (Fig. 1) was collected either as sequentially eluted fractions or as a single fraction. These fractions were freeze dried before application to an ODS column.

HPLC

The apparatus used was a Varian 5000 liquid chromatograph with computerised gradient making facility, Varian UV 50 variable wavelength detector (set at 230 nm) and an ODS-C_{18} column (25 cm × 5 mm; Shandon, Runcorn, Great Britain). Sample loop capacity was varied from 20 µl to 200 µl for analytical and preparative use, respectively. About 0.7 µg of Novo porcine proinsulin eluted with 29% acetonitrile and 71% 0.4 mol/l phosphate (pH 2.4) could be detected as a single peak of 10% of the optical density range 0–0.05 with the detector set at 230 nm. The mobile phase consisted of a water miscible organic solvent component together with an aqueous phosphate solution. The pump was programmed to generate binary mixtures of either acetonitrile and aqueous phosphate, or of methanol–acetonitrile (in preselected proportions) and aqueous phosphate.

Trypsinisation

This was carried out as described by Steiner *et al.*[4] on proteins initially separated by HPLC.

Polyacrylamide gel electrophoresis

This was carried out as specified by Schlichtkrull *et al.*[3].

RESULTS AND DISCUSSION

All of the gel chromatography subfractions b_1–b_6 of the proinsulin-containing

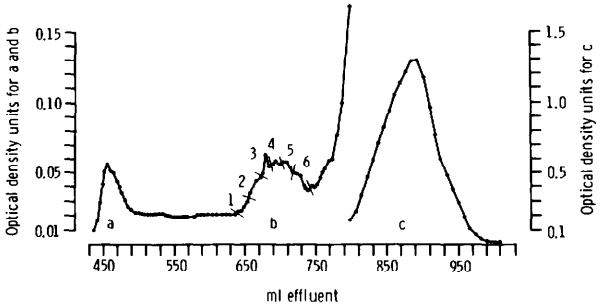

Fig. 1. Protein elution profile on gel chromatography of 250 mg crude porcine insulin. Optical density was monitored at 230 nm. The "b" component contains proinsulin and the "c" component contains insulin[4] (see Methods for details).

peak[4] (Fig. 1) yielded the same main five groups of proteins numbered I–V (Fig. 2) when re-chromatographed on HPLC with acetonitrile and 0.4 mol/l phosphate buffer, pH 2.4. A typical tracing of the optical density at 230 nm and the gradient applied is given in Fig. 2.

The proteins of group I were the only ones which eluted with 28 % acetonitrile; hence they were distinct from the group II proteins. The retention time of early impurities in group I was about 5 min in 29 % acetonitrile, which was very close to the solvent front in our system. Thus, the initial equilibration of the column with 28 % acetonitrile was useful in increasing the retention time of group I proteins, and served to prevent overlap with the solvent front.

Variation of buffer pH between 2.1 and 2.5, or addition of 7 mol/l aqueous urea to the samples to check the possibility of polymeric association of the different forms of the proteins[1,4] did not alter the sequential pattern of elution of proteins or

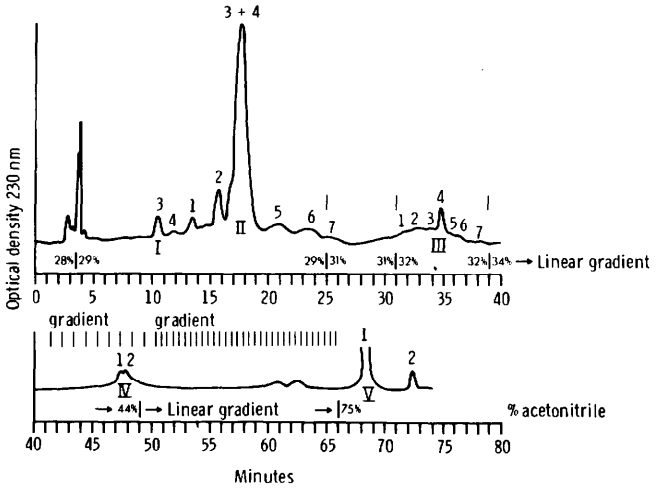

Fig. 2. Protein elution profile on HPLC of pooled fractions b_1–b_4 (as in Fig. 1) with acetonitrile and 0.4 mol/l phosphate pH 2.4. The changes in the acetonitrile proportion of the mobile phase are indicated. Distinct groups of proteins are designated I–IV and individual peaks in groups are given in Arabic numerals.

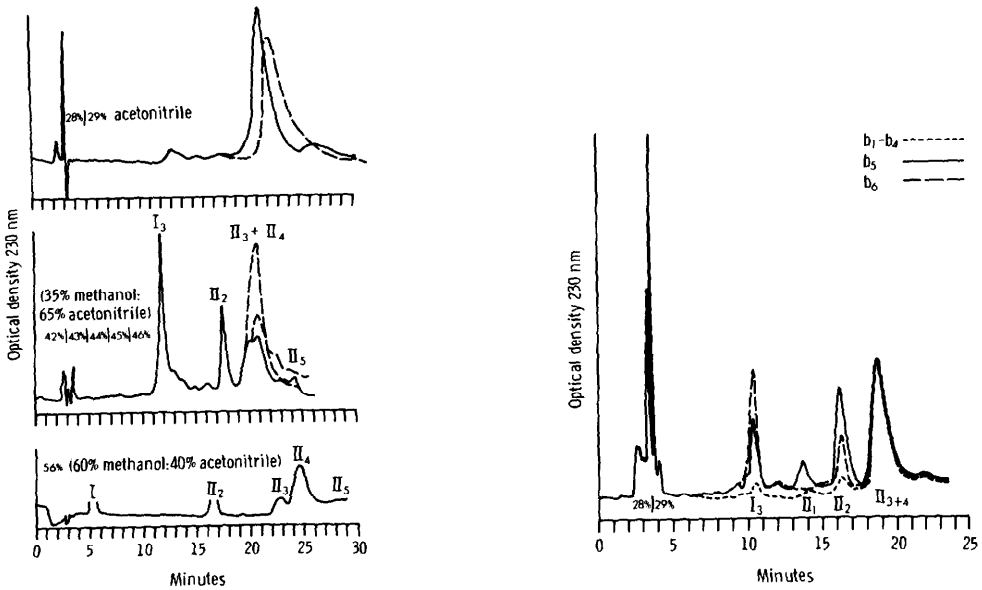

Fig. 3. Top: HPLC with acetonitrile and 0.4 mol/l phosphate (pH 2.4) of subfraction b_1 and of the Novo porcine proinsulin (---) (marker). When eluted together the marker proinsulin formed a slight shoulder on the main b_1 peak. Middle: HPLC with 42–46% of a mixture of 35% methanol–65% acetonitrile and 58–54% of 0.4 mol/l phosphate (pH 2.4) of subfraction b_1 and Novo porcine proinsulin (---). When eluted together, peaks II_3 and II_4 of b_1 and marker proinsulin overlapped. Bottom: Separation of peaks II_3 and II_4 with 56% of a mixture of 60% methanol–40% acetonitrile and 44% of 0.5 mol/l phosphate (pH 2.4).

Fig. 4. Variations in the relative proportions of group I and II main peaks in subfractions b_1–b_6 of porcine "proinsulin" (as in Fig. 1). The peptide profile of the pooled b_1–b_4 subfractions converted, after reconstitution in 0.2 mol/l phosphate, pH 2.4 and storage at −30°C, to that seen with b_5 and b_6 subfractions as shown in this figure (HPLC) as in Fig. 2).

the retention times. The pH value of 2,4 was selected to minimize deterioration of the column.

The choice of 0.4 mol/l phosphate was made by testing the elution characteristics of the proinsulin marker with 29% acetonitrile. The minimal phosphate requirement at pH 2.4 was about 0.3 mol/l, pH 2.4, but tailing of the peak was prevented by 0.4 mol/l phosphate.

Identification of the proinsulin peak in the groups of proteins I–V was achieved by using the Novo porcine proinsulin as a marker (Fig. 3). Care was taken that the marker proinsulin and the samples analysed were treated in exactly the same way before HPLC, *e.g.*, by freeze drying from a solution in 1 mol/l acetic acid when samples were analysed immediately after Sephadex gel chromatography; or by freeze drying, desalting by picration and reprecipitation with acetone–hydrochloric acid[9] after HPLC before rechromatography. This precaution was necessary lest proteins carried different charges when their retention times at a particular ionic strength were to be compared.

Better separation of the components of group II was achieved by replacing acetonitrile with mixtures of methanol–acetonitrile (Fig. 3). Thus 42% to 46% of a mixture of 35% methanol–65% acetonitrile and 58% to 54% of 0.4 mol/l phosphate (pH 2.4), respectively, separated II_2 from II_3 and II_4 which were still very close.

Whereas 56 % of a mixture of 60 % methanol–40 % acetonitrile and 44 % of 0.5 mol/l phosphate (pH 2.4) separated II_3 and II_4 reasonably well but caused significant spreading of II_2 and II_5. Therefore as a working procedure the peaks $II_{3/4}$ were collected initially from an acetonitrile gradient as in Fig. 2, and rechromatographed with 56 % of a 60 % methanol–40 % acetonitrile mixture and 44 % of 0.5 mol/l phosphate (pH 2.4) to separate II_4 which upon electrophoresis gave a band of similar mobility to that of porcine proinsulin marker.

Analysis of six separate batches of "b" component subfractions suggested that the proteins of groups I, II (peaks 1 and 2), V and possibly III and IV were spontaneous conversion products of peak II_4 which eluted identically to the porcine proinsulin marker (Fig. 3a and b). In two of the six batches of "b" component which were analysed, groups I and V proteins predominated while group II proteins were virtually absent. In one batch, when the subfractions were freshly dissolved in 0.2 mol/l phosphate pH 2.4, the amounts of I_3 and II_2 contained in b_1–b_4 was very small as compared to b_5 and b_6, but were raised (Fig. 4) after storing the fractions at −30°C. Also, the Novo porcine proinsulin was found to contain small amounts of impurities of the I, II_2 and V types. Lastly, after separation by HPLC, pooling, freeze drying, desalting and reprecipitation, rechromatography of the group I–IV peaks with the acetonitrile gradient as given in Fig. 2 revealed consistent contamination with a protein similar to that of group V, although main peak of group V did not appear to convert to another protein detectable by rechromatography by HPLC.

Trypsinisation of the separated proteins also supported the possibility that most of the peaks eluted on HPLC which were not proinsulin were indeed closely related to proinsulin. HPLC of the trypsinisation mixtures of each protein (Fig. 5) provided this evidence. The patterns of peptides which were released after mild or

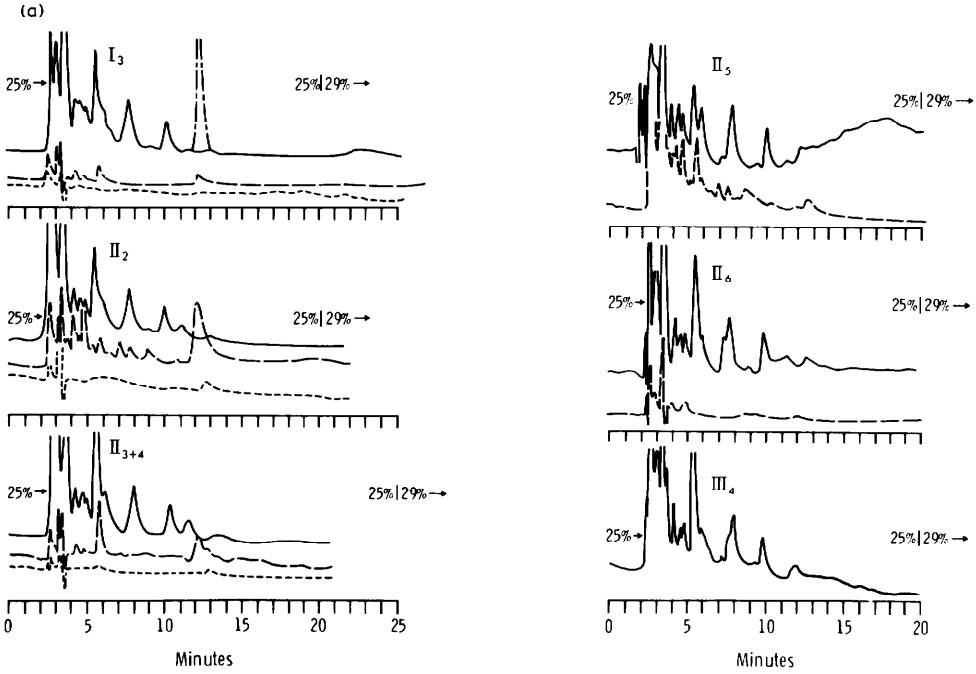

Fig. 5.

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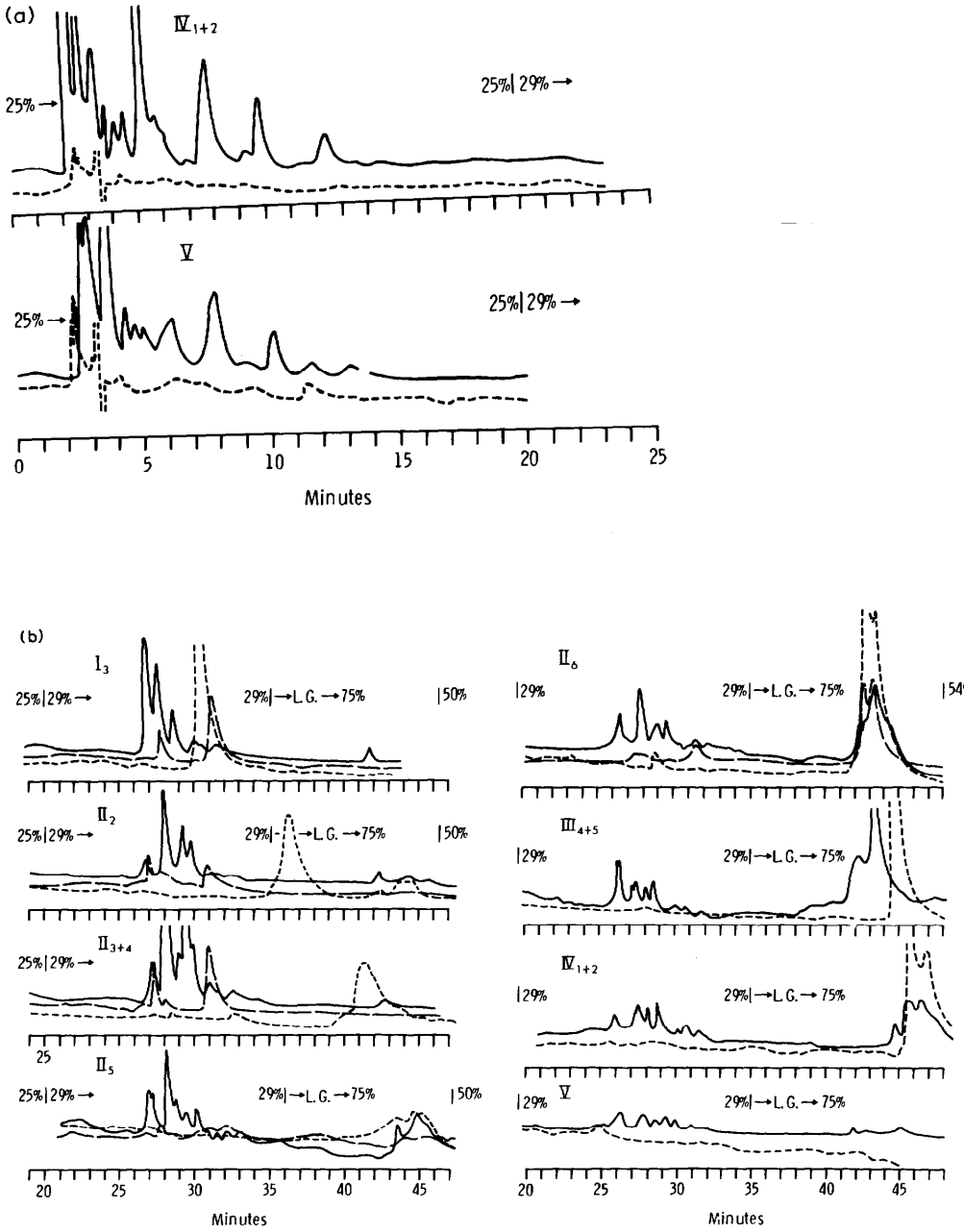

Fig. 5. Trypsin treatment at 37°C for 30 min[4] of the proteins I–V eluted by HPLC of crude porcine proinsulin or component "b" (as in Fig. 1). Optical density tracings at 230 nm on HPLC of the untreated protein (-----); after mild trypsinisation(——), after trypsinisation (———) and of Actrapid porcine insulin (———) (5a, 1). HPLC was carried out with an initial run with 25% acetonitrile–75% phosphate (0.4 mol/l; pH 2.4) for 20 or 25 min (series a); then acetonitrile was increased to 29% for 15 min (except in the case of II_{3+4} shown above), after which a linear gradient of 29–75% acetonitrile at a rate of 2% per minute was applied (series b).

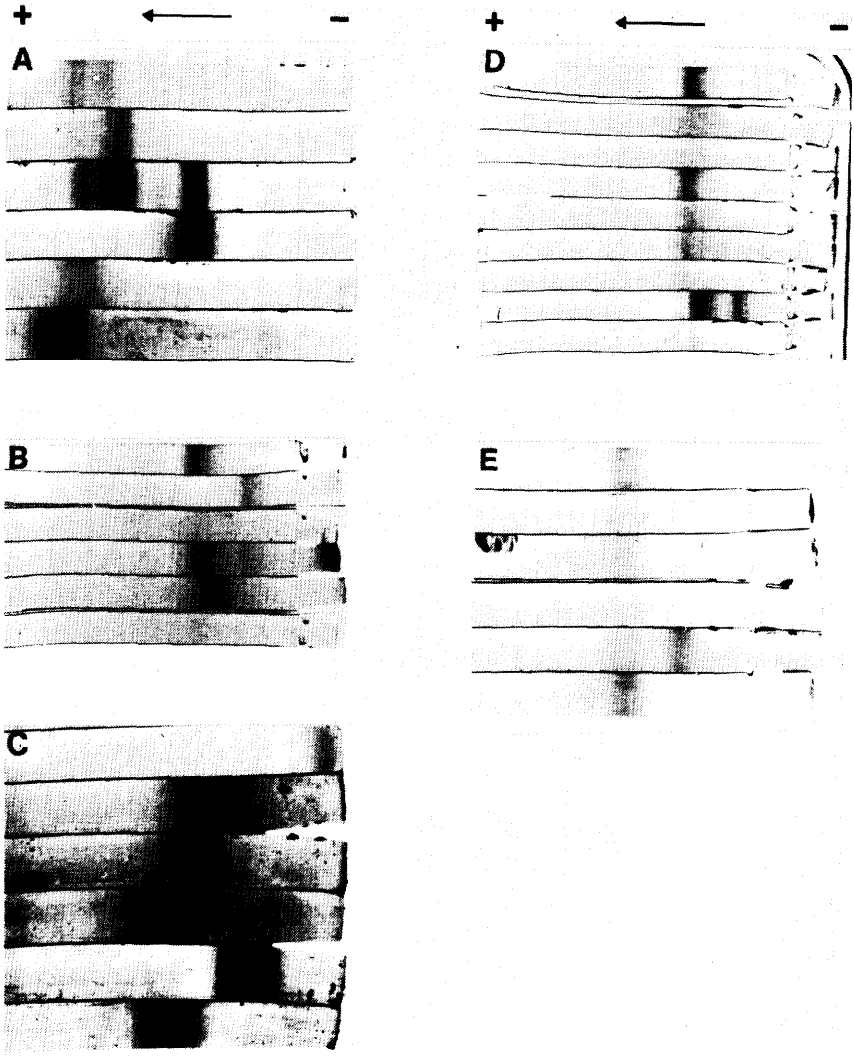

Fig. 6. Polyacrylamide gel electrophoresis of peaks eluted by HPLC of the crude "b" component subfractions illustrated in Fig. 2, and also of the "marker" Novo porcine proinsulin and "Actrapid" porcine insulin: A, 6 h at constant current of 3.0 A per gel; B,D,E, 4 h at constant current of 3.0 A per gel; C, 2 h for the group V proteins, otherwise 4 h of electrophoresis. The sequence is from top downwards, thus: A, I_3 + I* (see Note 1) (Fig. 5b), II (Fig. 5b), I–II_{1-5} (Fig. 4, b_1–b_4), II_4 (Fig. 3c), II_4 + Trypsin and III_4 + trypsin (Fig. 5b); B, porcine insulin* (see Note 2) (Fig. 5a), proinsulin marker (Fig. 3b), proinsulin marker + trypsin, I–V (Fig. 1), I* and III_4 + trypsin; C, (see Note 3) V (Fig. 1), II_2 (Fig. 4), II_2 (Fig. 3), I_3 (Fig. 4), III_4 + proinsulin marker (Fig. 3) and porcine insulin** (Fig. 5a); D, porcine insulin** (Fig. 5a), II_6 (Fig. 5b), II_6 + trypsin (Fig. 5a and b), III_4 (Fig. 5b), III_4 + trypsin (Fig. 5a and b), IV (Fig. 5b), IV + trypsin (Fig. 5a and b), I–II_{1-5} (Fig. 4, b_1–b_4) and II_2 (Fig. 5b); E, porcine insulin**, I_3 (Fig. 5b), I_3 + trypsin (Fig. 5a and b), II_2 + trypsin (Fig. 5a and b), II_4 + porcine insulin**, II_4 + porcine insulin** + trypsin. *Note 1:* Batch 6 b_{1-6} was found to consist of mainly I* (and V) type protein(s). This I* eluted slightly faster on ODS than I_3 obtained from other batches of b_{1-6}; but moved more slowly on electrophoresis. *Note 2:*** = "Actrapid"; *Note 3:* Excess protein applied on this gel shows the slow moving V band.

strong trypsinisation[4] were very similar for the proteins of groups I–V (Fig. 5a and b). The protein least vulnerable to trypsin appeared to be those in group V. On gel electrophoresis for 4 or 6 h proinsulin and peak II_4 had a mobility intermediate between that of insulin and that of the very slow moving group V proteins (Fig. 6). Porcine insulin and the proteins of groups I, III, IV and even II showed similar mobilities which indicated the limited selectivity of this system. Despite the apparent change in their elution characteristics from that of intact insulin during HPLC (Fig. 5), the mild trypsin conversion mixture of all types of proteins appeared as a single major electrophoretic band of comparable mobility to that of insulin (Fig. 6). This may be a reason why Steiner *et al.*[4] designated a protein obtained by ion-exchange chromatography of porcine proinsulin and of electrophoretic mobility similar to that of insulin before and after trypsinisation as a "non-(trypsin)-convertible" form of proinsulin.

The porcine insulins Actrapid and Monotard, as well as the main peak of crude porcine insulin obtained from Novo (which was used as the source of the "b" component analysed in this study) and the main peak of the "c" component obtained from this crude insulin (Fig. 1) all demonstrated a retention time of about 9 min by HPLC with 29% acetonitrile. This is very similar to that of the main peaks of the group I type of proteins. In all of these insulin preparations, an impurity with hydrophobic character comparable to that of the group V type of proteins, observed during HPLC of "proinsulin", was also detected. The Actrapid insulin could also be eluted in about 12 min with 25% acetonitrile and 75% phosphate buffer (pH 2.4) (Fig. 5a). Hence, trypsinisation may generate insulin and modified forms of the insulin molecule from respective precursors. Moreover the group I main peak, especially in those subfractions where this peak was the sole detectable entity with 28–29% acetonitrile elution, may be very similar to insulin structurally. Indeed protein II_2 may be one of the double chain conversion products of proinsulin[4].

CONCLUSIONS

Reversed-phase chromatography on ODS provides a simple and speedy method of purifying proinsulin from impurities and conversion products. The technique offers the sensitivity and selectivity required for studying the heterogeneity of proteins in general. In this work, the identification of the proinsulin peak was dependent on the use of a commercially purified preparation of prosinulin. The definite identification of each eluted proinsulin-related peak requires amino acid analysis, which may soon become widely available for application, especially with current developments in techniques of pre-column derivatisation of very small amounts of protein digests.

The results obtained suggest that insulin and proinsulin preparations may be persistently contaminated with spontaneous conversion products. Such products can be detected better with a gradient system of HPLC as described here, rather than by isocratic elution procedures so far reported by others. This is an important consideration in view of the potential antigenicity of therapeutic preparations which has been attributed to traces of contaminants.

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