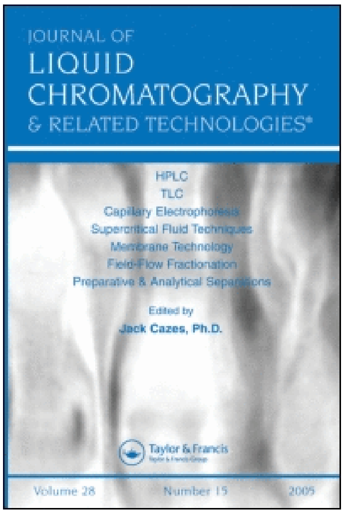

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A High Performance Liquid Chromatographic Assessment of the Isolation of Bovine Proinsulin and a Synthetic Proinsulin Fragment

John G. R. Hurrell[a]; Raymond J. Fleming[a]; Milton T. W. Hearn[b]
[a] Research and Development Division, Commonwealth Serum Laboratories, Victoria, Australia [b] MRC Immunopathology Research Unit, Otago University Medical School, Dunedin, New Zealand

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A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSESSMENT OF THE ISOLATION OF BOVINE PROINSULIN AND A SYNTHETIC PROINSULIN FRAGMENT

John G.R. Hurrell and Raymond J. Fleming,
Research and Development Division,
Commonwealth Serum Laboratories,
Parkville, Victoria 3052, Australia

and

Milton T.W. Hearn*,
MRC Immunopathology Research Unit,
Otago University Medical School,
P.O. Box 913, Dunedin, New Zealand

ABSTRACT

High performance liquid chromatographic techniques have been used for the analysis and purification of the bovine proinsulin C-peptide fragment 34-45 (H-Val-Glu-Gly-Pro-Gln-Val-Gly-Ala-Leu-Glu-Leu-Ala-OH) (I) prepared solid phase synthetic methods. Conventional open column chromatographic methods failed to resolve the desired dodecapeptide (I) from the des-Pro^9-undecapeptide (II), which constituted the major solid phase synthetic deletion product. On 5- or 10- μm microparticulate reversed phase columns, these peptides are readily resolved preparatively in less than twenty minutes with simple elution systems. The elution order is in accord with that expected on the basis of hydrophobic fragmental constant summation. These HPLC techniques have been extended to permit the analytical assessment of the isolation of bovine proinsulin and the proinsulin intermediates. The conversion of bovine proinsulin initially to the intermediates and finally to the desalanyl-insulin and the C-peptide on tryptic digestion can be followed by HPLC techniques which thus supplement alternative polyacrylamide disc electrophoretic methods.

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* To whom correspondence should be addressed.

INTRODUCTION

Proinsulin, the biosynthetic precursor of insulin, has been isolated and characterised from a wide variety of vertebrate species and its transformation, both in vivo and in vitro, to insulin and the interlocking C-peptide extensively documented (1-5). An analysis of the wide species variation found in the sequences of the C-peptides, compared to the conservation of structure shown by different insulins, suggests that the C-peptide may not have any specific endocrine function other than facilitating the folding of proinsulin to ensure the correct pairing of cysteinyl residues required to form the disulphide linkages of insulin (6-8). The C-peptide, however, when assayed immunologically, provides an important hallmark for following pancreatic dysfunction in vivo. The amino acid sequences of many mammalian C-peptide segments, including the bovine C-peptide (9), have been elucidated. Recently, high performance liquid chromatographic (HPLC) procedures have been applied to the analysis of a number of different insulins (10-12). In this paper, we wish to report the application of reversed phase HPLC for the analysis and purification of proinsulin related C-peptides. During part of these studies, related chromatographic procedures were found useful in following the isolation of bovine proinsulin from commercial crystalline insulin preparations.

MATERIALS AND METHODS

Synthesis of Bovine Proinsulin Fragment 34-45 (I)

Solvents and Reagents. Methylene chloride (AR) was obtained from BDH (Melbourne) and purified by passage down a column (6 x 90cm) of basic alumina (aluminium oxide 90 basic Merck) before use. Dimethylformamide (A.R. BDH Melbourne) was purified by sequential passage down acidic and basic alumina columns and stored over molecular sieve 4A. All protected amino acids were purchased from the Peptide Institute (Osaka, Japan). The α-amino functions of all amino acids were protected by the t-butyloxycarbonyl (BOC) group.

The γ-carboxyl function of glutamic acid was protected as the benzyl ester.

Synthesis. Chloromethylated polystyrene-divinyl benzene (2%) copolymer (100-200 mesh) was purchased from the Peptide Institute (Osaka, Japan) and esterified with Boc-alanine by refluxing in ethanol for 24 hours in the presence of triethylamine (13). The resulting resin contained 0.22mmole Boc alanine per gram.

Synthesis of the fragment was carried out in an apparatus similar to that described by Rosenblatt *et al*. (14). Coupling reactions were carried out in methylene chloride with a three fold molar excess of Boc-amino acid and N'N'-dicyclohexylcarbodiimide for each amino acid with the exception of leucine and glutamine. Leucine is only sparingly soluble in methylene chloride and hence dimethylformamide was used as the coupling solvent for this amino acid. Glutamine was introduced into the peptide by reaction of the p-nitrophenyl ester, in six fold excess, in dimethylformamide, with the peptide resin in the presence of 1-hydroxybenztriazole (15). The completeness of coupling after the addition of each amino acid was assessed qualitatively by the ninhydrin method (16). Incomplete couplings were repeated using dimethylformamide as the solvent. If a second reaction failed to complete the coupling, the unreacted α-amino groups were blocked by reaction with acetic anhydride in the presence of triethylamine (17). Cleavage of the completed peptide from the resin was by treatment with hydrogen fluoride in the presence of 1% anisole at $0^{o}C$ for 30 minutes. This was carried out by Dr G. Tregear of the Howard Florey Institute, Melbourne, using the procedure described by Stewart and Young (18).

Peptide Purification. The peptidic product was gel filtered on a column (54 x 2.4cm) of Sephadex G-25 (fine) in 0.5N ammonium bicarbonate. The leading edge of the main peak, which had no optical absorption at 280nm, was pooled and lyophilised. This lyophilised fraction was further purified by chromatography on a column (48 x 2.2cm) of carboxymethyl (CM) Sephadex C-50. A linear gradient was developed using 0.05M pyridine-acetic acid, pH 4.3

and 1.0M pyridine-acetic acid pH 4.3 at room temperature. The fractions collected (4.5ml) were monitored by the Fluorescamine procedure (19).

Partition chromatography of the crude synthetic peptide mixture (50mg) was carried out on Sephadex G-25 (fine) (column dimensions 48 x 2.2cm) equilibrated in the aqueous phase of the BAW buffer (n-butanol-acetic acid-water, 4:1:5) and eluted with the organic phase at a flow rate of 0.2ml/min. The peptides were detected fluorometrically via the fluorescamine reaction.

Chemical Characterisation. Peptides were hydrolysed from the resin using the HCl-propionic acid procedure (20). Acid hydrolysis of the peptides was in 5.7N HCl for 24 hours at 110°C *in vacuo*. Amino acid analyses were carried out using a Beckman 121M Amino Acid Analyzer. The resin-bound peptide was sequenced by Dr L. Sparrow of the CSIRO Division of Protein Chemistry, Melbourne using an LKB Solid-Phase Peptide Sequencer 4020. Thin-layer chromatography was carried out on silica gel plates (Merck, Kieselgel 60) in the following solvent systems: PEAH, Pyridine-ethylacetate-acetic acid-water (50:50:10:30), BAWP butanol-acetic acid-water-pyridine (60:12:48:40).

Isolation and Purification of Bovine Proinsulin.

Bovine proinsulin was isolated using modification of the ion-exchange method of Steiner *et al* (1) from first crystals of bovine insulin supplied by the Production Division Commonwealth Serum Laboratories, Parkville. Following gel chromatography on Sephadex G-50 (fine) (column dimensions 120 x 3cm) using a 1.0M acetic acid eluant, the pooled, lyophilised proinsulin-rich, as indicated by polyacrylamide electrophoresis, fractions (8g) were rechromatographed on a CM-cellulose column (column dimensions 53.5 x 5.4cm) with initially 0.01M citrate, pH 5.50 containing 7M urea. After elution of peak B, 0.2M NaCl was added to the eluting buffer. Isolated components from the ion exchange columns were dialyzed against 0.1M acetic acid, lyophilised and then examined by disc electrophoresis (21), in 10% polyacrylamide gels, after being dissolved in 0.1M Tris-o.001M EDTA pH 7.4 containing 7M urea.

Isolated proinsulin was subjected to tryptic digestion as confirmation of identity. TPCK-treated trypsin (Worthington lot 33J812) was added to a solution of proinsulin in 0.1M NH_4 HCO_3, pH 8.2, to a level of 1% w/w. Digestion was carried out at 37°C and samples taken at 5, 10, 20, 30 and 90 minutes, were lyophilised, taken up in tris-urea buffer and examined by disc electrophoresis.

High Performance Liquid Chromatography (HPLC) All solvents were AnalaR grade. Methanol and acetonitrile, supplied by Fischer Scientific Co. were purified as reported earlier (22). Orthophosphoric acid was obtained from May and Baker Ltd. Water was deionised by reverse osmosis and distilled. A Waters high performance liquid chromatography system was used which included two M6000A solvent delivery units, an M660 solvent programmer and a U6K universal liquid chromatographic injector, coupled to a M450 variable wavelength UV monitor and a Rikadenki dual channel chart recorder. Sample injections were made with a Pressure-Lok liquid syringe, Series B110 from Precision sampling (Baton Rouge, La, U.S.A.). Solvents were filtered using a pyrex filter holder (Millipore Corp. Bedford, Mass. U.S.A.) and samples with a Swinney Filter (AP2500 Filters, Millipore Corp.). All chromatograms were carried out at room temperature (ca. 20°). All peptides were made up in the eluting solvent. Bulk solvents were degassed separately and the appropriate mobile-phase prepared and equilibrated as reported previously (22). All columns were equilibrated to new mobile phases for at least 30 minutes and after gradient elutions, the gradient was reversed and the columns re-equilibrated for 30 minutes after initial conditions were reached. Flow rates were maintained between 1.0ml/min. and 4.0ml/min. as indicated in the text. Detection of the peptides was usually carried out in the range of 205-220nm, depending on the nature of the sample and mobile phase. After semi-preparative chromatographic separations, the pH of the eluants was adjusted to ca. pH 7.0, and the appropriate

TABLE

Amino Acid Compositional Data for the Bovine C-Peptide Fragment (I)

	Theoretical	Resin bound peptide	Crude cleaved peptide	After G-25 chromatography	After CM-Sephadex chromatography	After μC_{18} chromatography
Alanine	2	2.00	2.00	2.05	2.12	2.07
Valine	2	2.00	2.02	2.02	2.09	2.06
Glycine	2	1.86	1.90	1.91	1.98	1.95
Leucine	2	1.67	1.70	1.82	1.87	1.91
Proline	1	0.24	0.26	0.42	0.51	0.94
Glutamic Acid	3	2.42	2.32	2.99	3.09	3.10

fractions were concentrated by evaporation under reduced pressure, and analysed by amino acid analysis and analytical HPLC.

RESULTS AND DISCUSSION

The synthesis of the bovine proinsulin fragment 34-45 (H-Val-Glu-Gly-Pro-Gln-Val-Gly-Ala-Leu-Glu-Leu-Ala-OH) (I) was carried out by standard solid phase techniques using t-BOC-alanine substituted polystyrene-2% divinylbenzene resin (220μmol/g). Acetylation of unreactive α-amino groups present after a second repeated coupling step (17) was required after residue 4,$(Leu)^4$, only. The cleavage of the peptide from the resin was carried out for a short time and at low temperature to reduce the reaction of the γ-carboxyl groups with anisole in the presence of HF (23,24). The peptide was recovered at 82% of the theoretical yield.

Although the synthesis of the bovine proinsulin fragment 34-45 (I) appeared to proceed smoothly as assessed by the ninhydrin test (16), amino acid analysis of the resin bound peptide and the crude, cleaved peptide indicated a low level of proline (Table). Solid-phase sequence analysis of a sample of the support bound peptide clearly indicated that proline at residue 9 (residue 37 of bovine proinsulin) was deleted in a large proportion of the peptide chain. Figure 1 shows a tlc plate of the PTH amino acids isolated during the sequence analysis. The lane corresponding to cycle 9 indicates that both glutamine (as evidenced by the presence of both glutamine and glutamic acid) and proline were found at residue 9. As well as partial deletion at residue 9 the tlc plate shows that a preview of following residues is seen at cycles 7, 8 and 11 suggesting that incomplete coupling occurred at these steps during the synthesis. These results suggested that the crude product, obtained from the solid phase synthesis, contained the desired bovine proinsulin fragment 34-45, together with contaminating amounts of des-Glu^{11}, des-Pro^9, des-Gln^8 and des-Val^7 derived peptides.

The crude peptide was chromatographed on a Sephadex G-25 (fine) column in 0.5N NH_4HCO_3. The elution profile is seen in Figure 2.

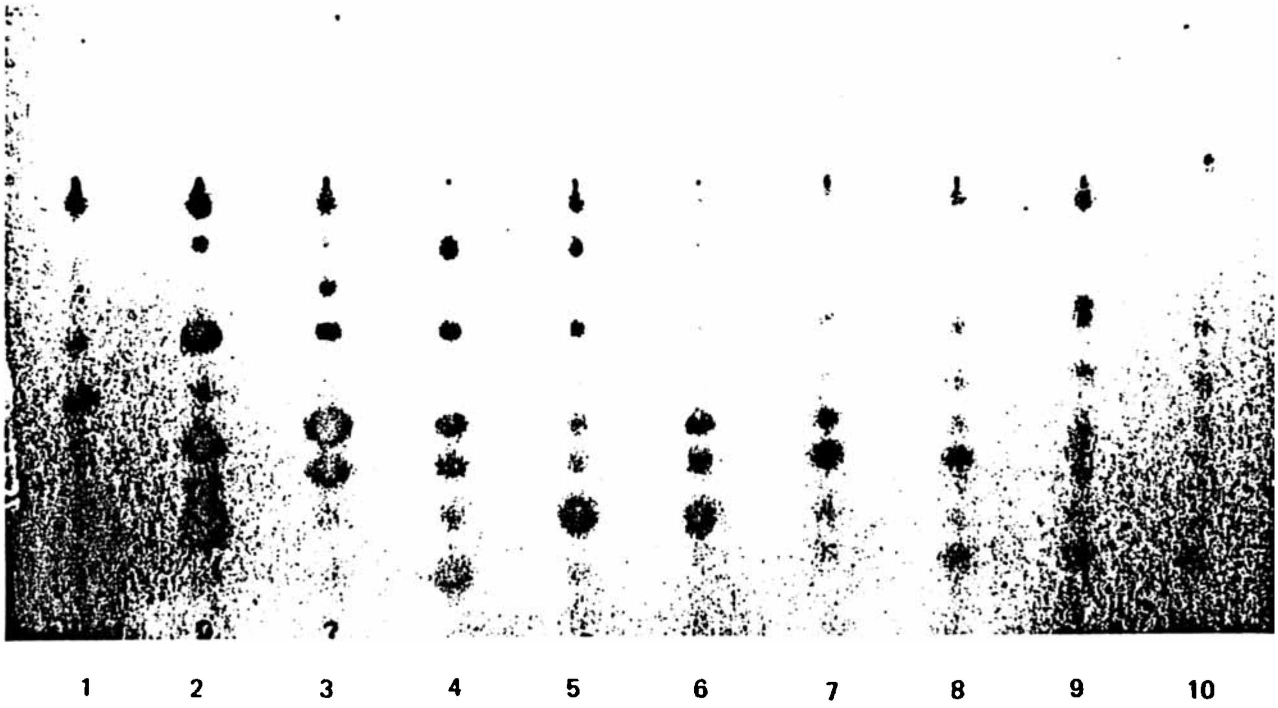

Figure 1. Tlc plate of PTH-amino acids recovered from solid-phase sequencer during analysis of resin-bound peptide fragment 34-45 (I) of bovine proinsulin, before HF cleavage. The number lanes indicate the sequencer cycle number and the new amino-terminal residues indicated in each lane are: 1 valine; 2 glutamic acid; 3 glycine; 4 glutamic acid and proline; 5 glutamine; 6 valine; 7 glycine. It is not possible to clearly distinguish the new amino-terminal residue in lanes 8, 9, and 10.

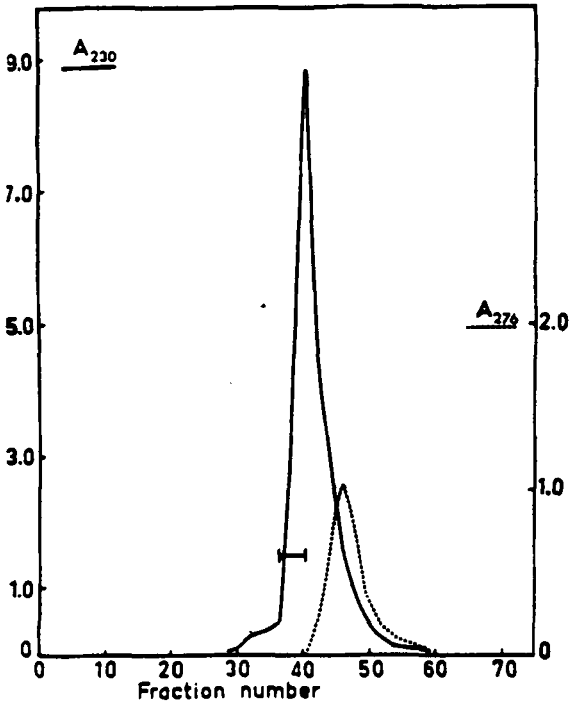

Figure 2 Elution profile for the gel filtration of the crude peptide mixture on a Sephadex G-25 (fine) column (54 x 2.4cm) equilibrated and eluted with 0.5N ammonium bicarbonate. The fractions indicated by the bar were pooled and lyophilised.

The trailing edge of the peak contained material which absorbed light at 280nm, suggesting this contained anisolated peptides. The leading edge was lyophilised and further purified on a column of CM-Sephadex (C-50) developed with a linear pyridine-acetic acid gradient. The elution profile is seen in Figure 3. The major peaks were pooled and lyophilised. The amino acid analyses for the pooled fractions are seen in the Table. These analyses suggested that the final CM-Sephadex fractionated material (which was obtained in 22% yield) contained approximately 50% proline deletion peptides.

Sensitive and rapid techniques for HPLC analysis and purification of underivatised peptides, obtained from solid phase synthesis

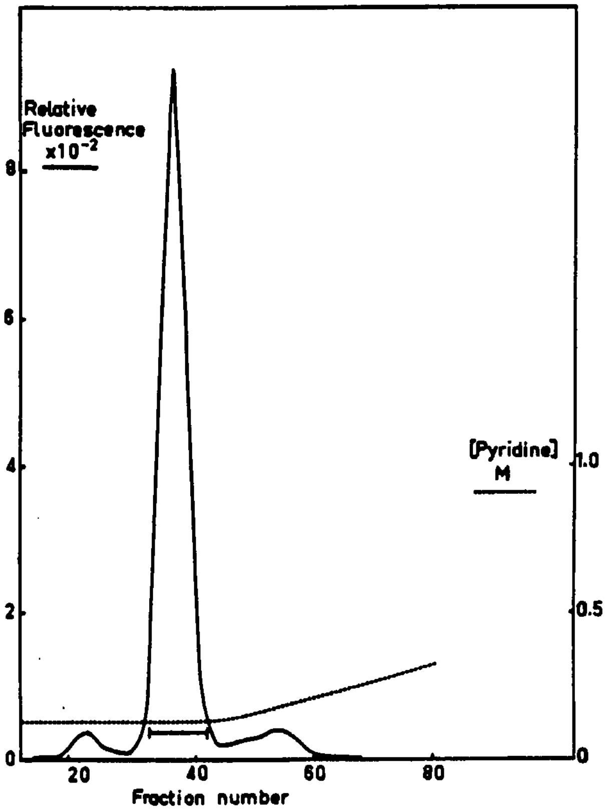

Figure 3 Elution profile of peptide, previously isolated by gel filtration (Figure 2), on CM-Sephadex C-50 using a pyridine gradient at pH 4.3. The main peak was pooled as indicated and lyophilised.

have recently been described (10,22,25,26). Besides allowing the assessment of homogeneity of partly purified peptides, these methods have the capability to be used preparatively. Purification of peptides obtained from solid phase synthesis has traditionally involved open column chromatography procedures based on gel permeation, ion-exchange, partition or adsorption methods or counter current distribution techniques. Depending on the nature of the components all of these techniques can have low separation efficiencies (as observed above) and are relatively time consuming. Because of the poor resolution frequently shown by these methods with mixtures of closely related peptides, as can be obtained in

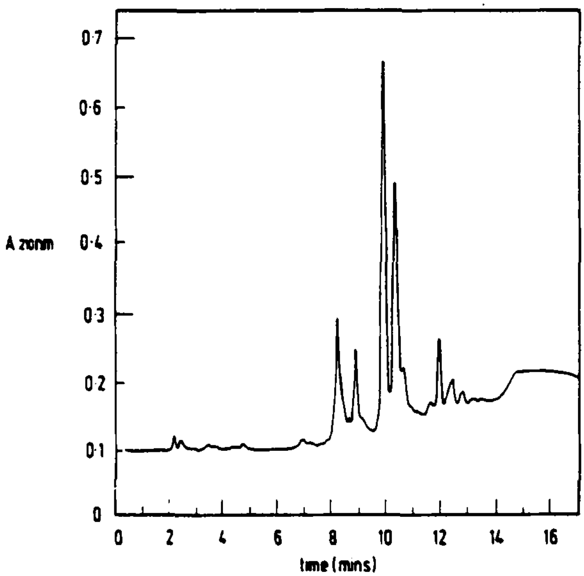

Figure 4 Analytical gradient elution profile of crude C-peptide fragment (I) on a μBondapak-C_{18} column at a flow rate of 2mL/min. A linear 10 minute gradient from acetonitrile-water-phosphoric acid (10:90:0.1%) to acetonitrile-water-phosphoric acid (50:50:0.1%) was started 1 minute after injection of the sample.

solid or solution phase synthesis due to deletion errors, the isolated fractions need to be monitored by analytical methods to ascertain purity. Generally, thin layer and paper chromatography or electrophoretic methods have been used to monitor the homogeneity of peptide synthesis. It is clear from recent studies with fully porous reversed-phase packings and ion-pairing elution strategies that HPLC techniques are complementary to methods conventionally used, having the important advantages of high speed, good recoveries and level of resolution greater than found with open column chromatography.

The analytical gradient HPLC chromatogram of the CM-Sephadex purified synthetic bovine C-peptide (I) using a μBondapak-C_{18} column at a flow rate of 2mL/min is shown in Figure 4. The gradient was generated using the hydrophilic phosphate approach

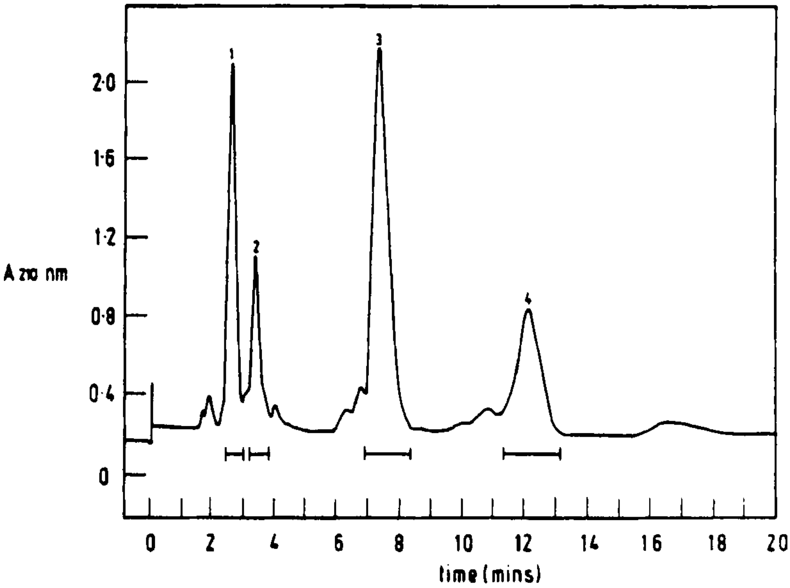

Figure 5 Semi-preparative separation of the CM-Sephadex fractionated C-peptide fragment (I) on a μBondapak-C_{18} column at a flow rate of 2mL/min. using a mobile phase of acetonitrile-water-phosphoric acid (20:80:0.1%). Fractions were pooled as indicated by the bars; peak 3, des-Pro^9-peptide (II); peak 4, C-peptide fragment (I).

from acetonitrile-water-phosphoric acid (10:90:0.1% to 50:50:0.1%). Figure 5 shows the semi-preparative separation of this crude peptide under isocratic conditions with a mobile phase of acetonitrile-water-phosphoric acid (20:80:0.1%) on 10μm Bondapak-C_{18} columns. Following recovery of the eluted fractions (82% overall yield) amino acid analyses and analytical HPLC confirmed that the desired dodecapeptide (I) eluted with a retention time of ca. 12 minutes whilst the des-Pro^9-peptide (II) eluted ca. 7 minutes. Similar resolution of the crude synthetic product, as well as the CM-Sephadex chromatographed fractions, can be achieved with 5μm Lichrosorb-ODS columns and a mobile phase of methanol-water-phosphoric acid (50:50:0.5%).

Due to the poor resolution obtained with the open column gel and ion-exchange systems, partition chromatography (27) of the

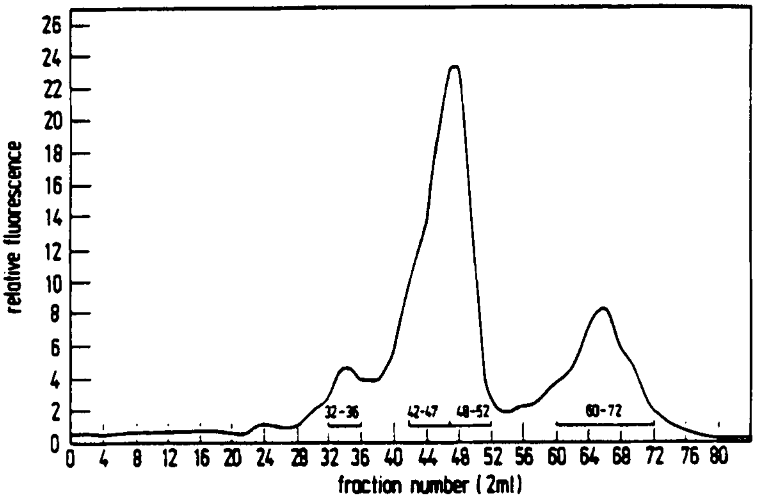

Figure 6 Partition chromatography of the crude C-peptide fragment (I) on a Sephadex G-25 (fine) column (48 x 2.2cm) equilibrated in the aqueous phase of a BAW buffer (n-butanol-acetic acid-water, 4:1:5) and developed with the organic BAW phase at a flow rate of 0.2ml/min. Fractions were pooled as indicated by the bars.

crude peptide (I) (50mg) was attempted on a Sephadex G-25 (fine) column, equilibrated in the aqueous phase of n-butanol-acetic acid-water (4:1:5) and developed with the organic phase. The chromatogram is shown in Figure 6. Fractions were collected as indicated, the bulk of the recovered peptides were contained in the major peak (28.5mg). The analytical HPLC separation of the leading edge of this peak (fractions 42-47) is shown in Figure 7. This indicates contamination of the C-peptide fragment (I) by des-Pro^9-peptide (II) although most of the other deletion peptides have predominantly been removed on partition chromatography. Although the elution order of closely related peptides on both partition and porous microparticulate reversed phase supports can be explained in terms of hydrophobicities of the amino acid residues, the superior efficiency and selectivity of the HPLC system is clearly apparent in this care.

Gel chromatography of the first crystals of bovine insulin

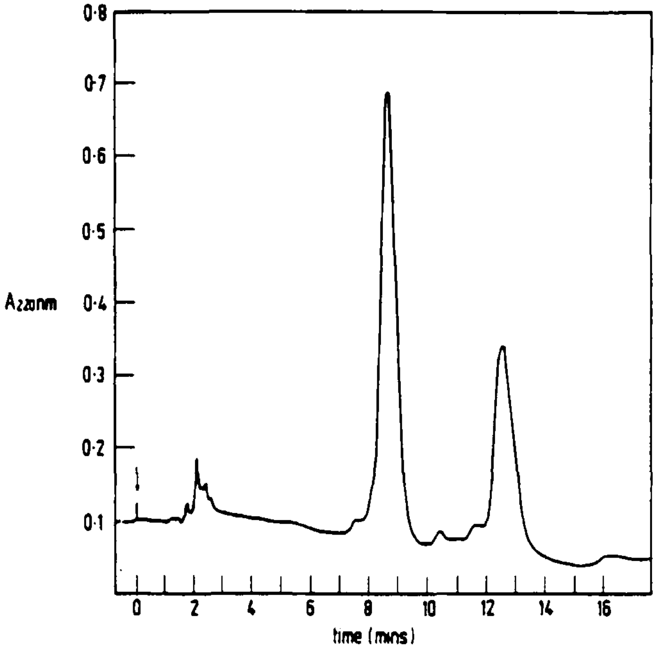

Figure 7 HPLC elution profile of the partition chromatography purified C-peptide fragment (I) from pooled fractions 42-47. The chromatographic conditions were: column: Lichrosorb ODS; mobile phase methanol-water-phosphoric acid (50:50:0.5%); flow rate 2mL/min.

partially resolves the minor components, including the proinsulin fraction. The procedure of Steiner et al. (1), for the isolation of proinsulin employs an initial gel filtration step on a Sephadex G-50 column with 1N acetic acid to separate the bulk of the insulin from the proinsulin which is usually present at 1-5% of the sample weight. When chromatographed on a Sephadex G-25 (fine) under the same elution conditions, the peak containing the proinsulin was seen as a shoulder on the leading edge of the insulin peak (Figure 8). This was confirmed by disc gel electrophoresis, and the fractions containing proinsulin pooled. After lyophilisation this pool was taken up in citrate buffer and applied to a carboxymethyl cellulose (CM) column. Two peaks, A & B

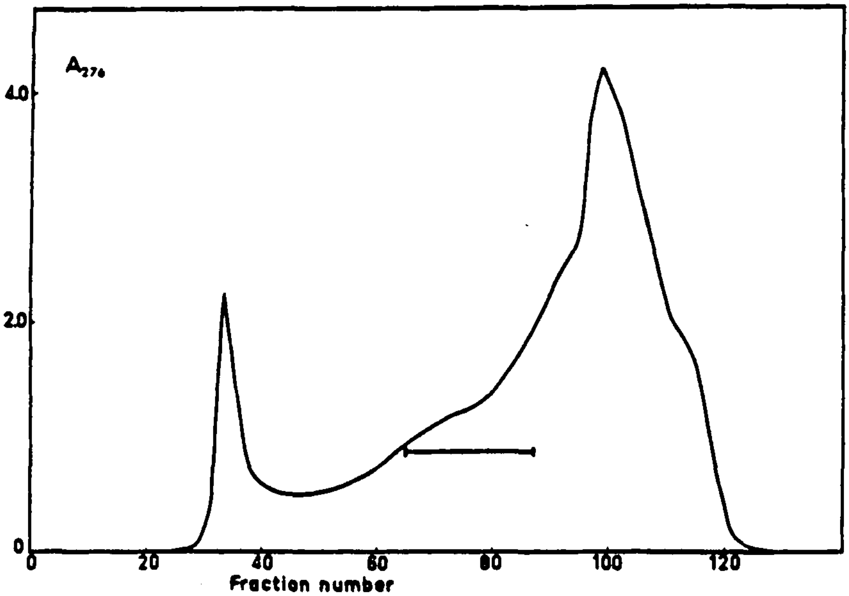

Figure 8 A typical elution profile for the separation of the components of the first crystals, formed in the isolation of bovine insulin, on a Sephadex G-50 (fine) column (120 x 3cm) in 1.0N acetic acid. The fractions containing proinsulin (as indicated by disc gel electrophoresis) were pooled and lyophilised.

(Figure 9) were eluted on washing the column with the initial buffer. Proinsulin was then eluted, with other components, on addition of 0.2M NaCl to the eluting buffer (Peak C). This is a departure from the earlier procedure where the NaCl is applied immediately after the emergence of Peak A. Peak C was rechromatographed (after desalting) on a column of Diethylaminoethyl Cellulose (DEAE). Proinsulin was eluted in peak 4 on elution with a continuous NaCl gradient (Figure 10). Disc gel electrophoresis of peaks 3 and 4 (Figure 11) indicates that peak 3 contained the intermediates from the conversion reaction of proinsulin to insulin and peak 4 contained proinsulin with a minor amount of intermediates as contaminant.

The reversed-phase HPLC of purified bovine proinsulin on a μBondapak-C_{18} column is shown in Figure 12. Under the same

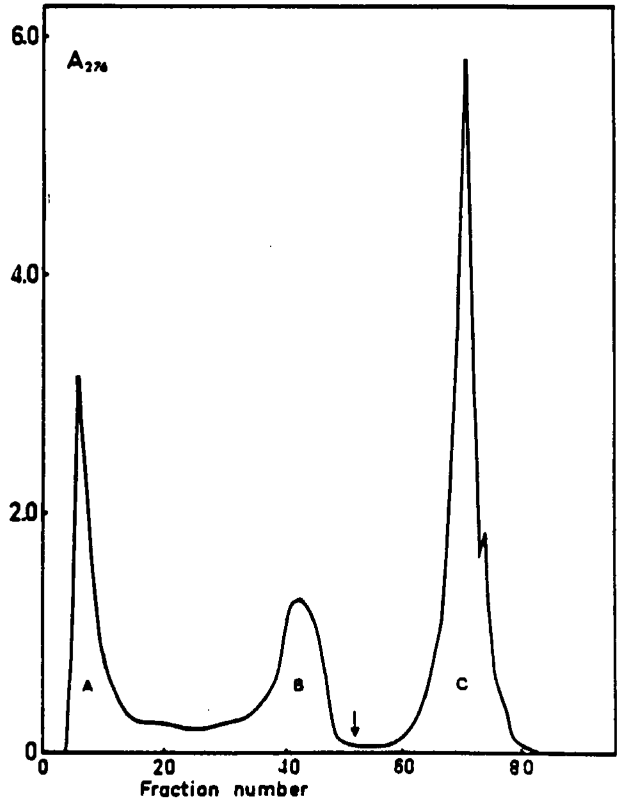

Figure 9 Profile obtained for crude proinsulin (isolated by gel filtration on Sephadex G-50 (fine)) on elution from a CM-cellulose column (53.5 x 5.4cm) with, initially, 0.01M citrate, pH 5.50 containing 7M urea. After the elution of peak B, 0.2M NaCl was added to the eluting buffer (indicated by arrow). The column loading was 8.0g isolated from multiple runs of first crystals on Sephadex G-50 columns.

chromatographic conditions and column bovine insulin elutes with a retention time ca. 8.5min., cf. bovine proinsulin ca. 12min. Using these HPLC methods, it is possible to follow the conversion of bovine proinsulin to insulin by tryptic digestion and thus this method provides a rapid alternative to the conventionally used polyacrylamide gel electrophoretic techniques. In contrast to the *in vivo* conversion of proinsulin to insulin, tryptic digestion

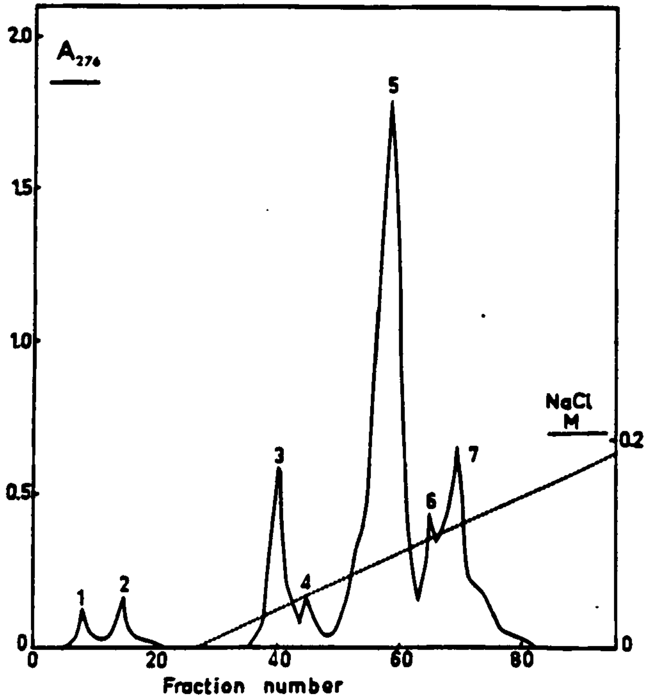

Figure 10 Gradient elution profile of peak C from CM-cellulose column on a DEAE cellulose column (53 x 5.4cm). Sample was applied in 0.02M Tris-HCl pH 7.4 containing 7M urea. A NaCl gradient, up to 0.2M, was developed as indicated.

results in the production of desalanyl-insulin (28). Bovine insulin and desalanyl-insulin are not separated by conventional polyacrylamide gel electrophoresis at pH 8.9 or reversed phase HPLC under the above low pH phosphate conditions but can be resolved using hydrophobic ion-pairing reagents (26,29) in the mobile phase. The disc gel electrophoresis patterns for the tryptic digestions of the protein in peaks 3 and 4 from the DEAE cellulose column (Figure 10) are seen in Figure 11; gels numbered 1 and 2 being peaks 3 and 4 respectively before the addition of trypsin. As the digestion proceeded the intermediates (peak 3) converted to desalanyl-insulin. The digestion was essentially complete at 90 minutes. Gel number 4 in Figure 11 shows that after only a 5 minute digestion, most of the

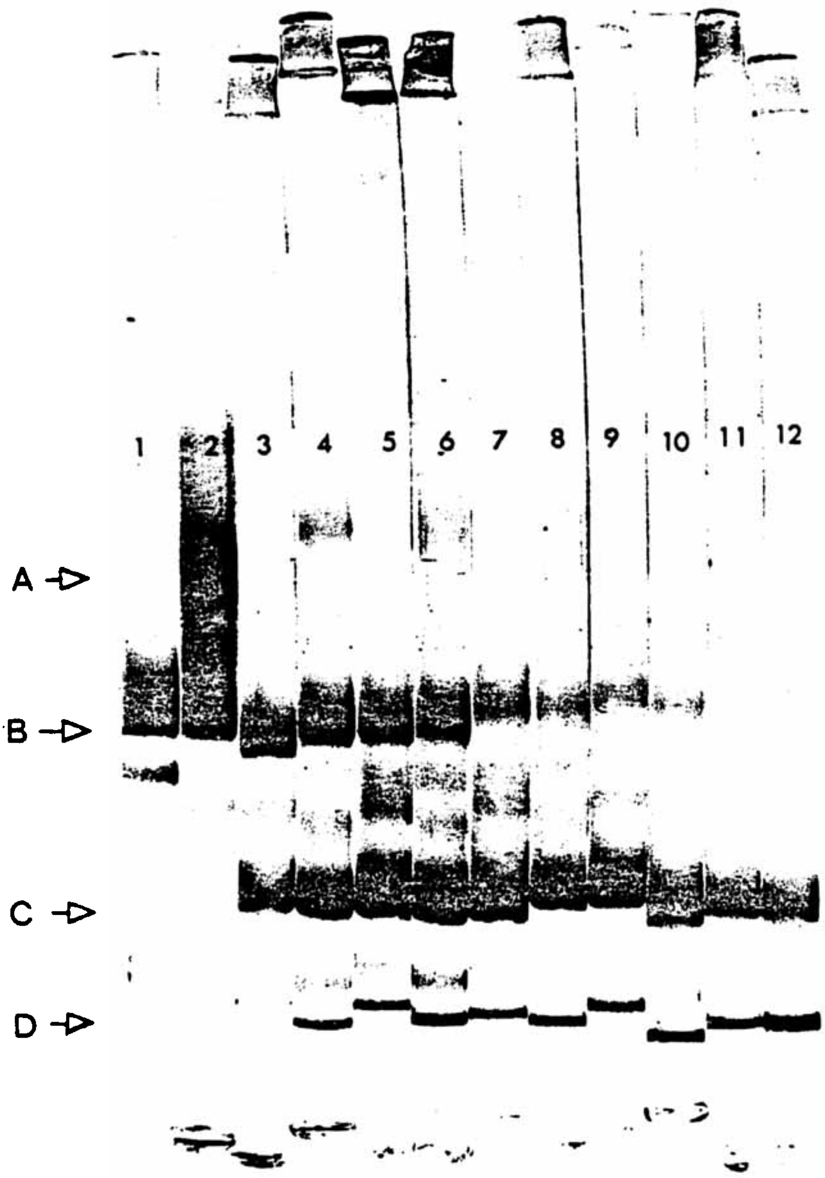

Figure 11 Polyacrylamide disc electrophoresis gels showing time course of a tryptic digestion of protein in peaks 3 and 4 (Figure 12). Gels 1,3,5,7,9 and 11 show peak 3 at 0,5,10,20,30 and 90 minutes respectively and gels 2,4,6,8,10 and 12 show peak 4 at the same times. Band A is proinsulin, Band B is the intermediates, Band C is desalanyl-insulin and Band D is the C-peptide.

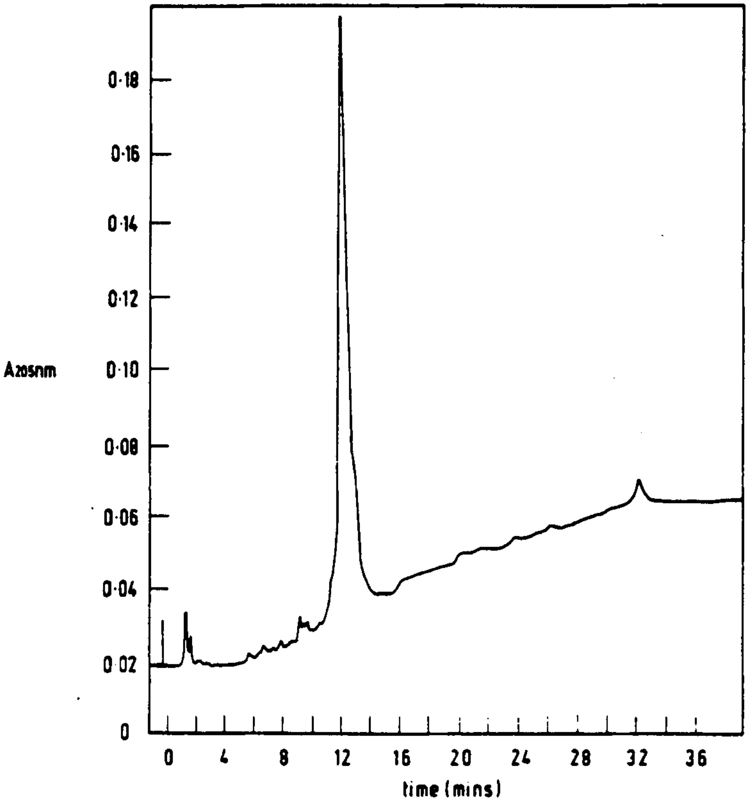

Figure 12 Analytical elution profile obtained with bovine proinsulin using a 30 minute linear gradient generated from acetonitrile-water-phosphoric acid (10:90:0.1% to 75:25:0.1%). Column: μBondapak-C_{18}, flow rate 2mL/min. Under the same chromatographic conditions and column bovine insulin elutes with a retention time of 8.5 minutes.

proinsulin from peak 4 had converted to intermediates and desalanyl-insulin. After 20 minutes (gel 8) there was no proinsulin remaining in the digestion mixture and little intermediates. At 90 minutes all the intermediates had been converted to desalanyl-insulin (gel 12). The fast moving band which increased in intensity as the digestion progressed is the connecting C-peptide, released during the proinsulin to insulin conversion. These results parallel earlier reports (1,30) for the enzymatic digestion of porcine and bovine proinsulin.

The advantages of reversed phase HPLC over conventional open column chromatography are clearly demonstrated by comparison of the different purification procedures for the crude peptide mixture containing the bovine C-peptide fragment 34-45 (I) (Figures 4-7). Furthermore hydrophobic fragmental constant summation (27,29) can be used for unprotected peptides of this type to accurately predict the elution order on microparticulate reversed phase columns.

The HPLC methods reported in this paper also provide a rapid and useful analytical adjunct to polyacrylamide gel electrophoresis for following the purification of larger insulin related polypeptides. The application of these HPLC techniques to the purification of insulins and related polypeptide and precursors from animal pancreases will be reported elsewhere.

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