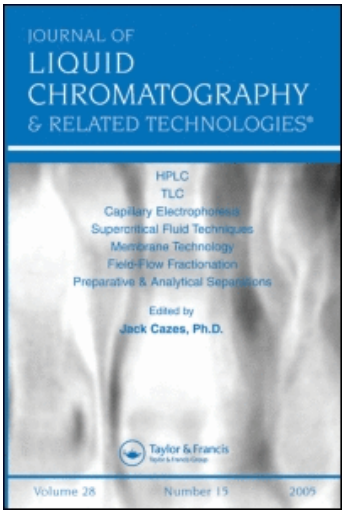

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PREPARATIVE ISOLATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF HUMAN INSULIN B CHAIN PRODUCED IN ESCHERICHIA COLI

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ABSTRACT

A simple method has been developed for the analytical and preparative purification of human insulin B chain from recombinant origin. Three solvent systems: acetonitrile, isopropanol and methanol, were studied to determine their capacity to resolve the insulin B chain from a mixture of cyanogen bromide generated bacterial peptides. Using a μBondapak C18 column, it was possible to resolve the insulin B chain in all three systems. On a preparative scale, using a PrePak 500 C18 column with the isopropanol system, it was possible to purify insulin B chain and to obtain a 95% protein recovery.

INTRODUCTION

Biosynthetic human insulin obtained by recombinant DNA techniques, represents a safe and important source of insulin for the treatment of

insulin-dependent diabetics. Since injection is at this time one of the few methods used for the administration of pharmaceutical proteins, the purification processes for these polypeptides must be designed to meet the stringent requirements of purity for solutions to be injected (1).

In the design of the recuperation procedure for a protein product, a number of parameters should be considered. These include the degree of purity desired, the physical and chemical properties of the product and its contaminants, and finally their cellular localization, for they may be found either in the cytoplasm, the periplasm or the growth media.

A purification process is usually a cascade of unitary operations, some of which may be repeated several times. Unfortunately, there is a loss of product in each operation and these losses grow geometrically with each step. For this reason, it is important to increase the yield in each step, or decrease the number of steps required (2).

The scale-up of a purification process from laboratory to pilot plant is not a direct process. It requires the knowledge of the physicochemical behavior of the product and its contaminants. Also it should be considered that the procedures used in the laboratory-scale purification process are not always convenient in other scales (3).

The method reported here for the purification of human insulin B chain from a bacterial peptide mixture, is based on the use of ion pair forming agents (4). In this case, formic acid and triethylamine were chosen since they modify the retention times of the peptides to be purified, allowing the resolution of human insulin B chain.

The criteria considered to develop this method were: capacity to purify the human insulin B chain from most of the bacterial contaminant peptides, low cost, flexibility for scaling up and the use of a volatile solvent system.

MATERIALS AND METHODS

High Performance Liquid Chromatography

Equipment.

Analytical chromatography was performed using the following equipment from Waters: M-45 and M-590 solvent delivery systems, automated gradient controller, U6K manual injector, Z module radial compression separation system and a M-481 L.C. spectrophotometer. Also, an OmniScribe recorder from Houston Instruments was utilized.

The preparative chromatography was performed using the Prep LC/System 500 A Liquid Chromatograph from Waters with a M-481 L.C. spectrophotometer with a preparative cell, and the OmniScribe recorder from Houston Instruments.

The columns used were: a μBondapak C18 cartridge (8 mm x 100 mm) from Waters, a metallic column (4.6 mm x 150 mm) packed with 1 gram of Pre-C18 preparative packing, and a PrepPAK 500 C18 cartridge (57 mm x 300 mm) from Waters.

Reagents

HPLC/Spectro grade methanol, isopropanol, and acetonitrile from Merck. Formic acid, sodium sulfite, urea and triethylamine from J. T. Baker. Cyanogen bromide and sodium tetrathionate from Sigma Co. Guanidinium chloride from Pierce. Insulin B chain derived from porcine (Sigma I-3505) was used as a standard.

Polyacrilamide Gel Electrophoresis (PAGE)

Slab gel electrophoresis in the presence of sodium dodecyl sulfate was performed as described by Laemmli (5).

Isolation and Purification of Inclusion Bodies

The method used was the one reported by Flores et al. (6).

RESULTS AND DISCUSSION

Our group has reported the construction of a recombinant plasmid where the human insulin B chain is produced as the carboxyl terminus of a fusion protein with the amino fragment from phage lambda cI protein. This hybrid protein is aggregated intracellularly as inclusion bodies, which after cell lysis are purified by a simple differential centrifugation step (6). The inclusion bodies are dissolved in 70% formic acid and treated with cyanogen bromide to release the insulin B chain (7). The formic acid is evaporated and the bacterial peptide mixture is dissolved in 8M guanidinium chloride. Sodium sulfite and sodium tetrathionate are added to protect sulfhydrile groups in the B chain from final oxidation, transforming them to sulfonate radicals or Bunte salts (8,9). The peptide mixture is dialysed, and urea is added to 8M. The resulting solution is diluted 1:1 with 5% formic acid, prior to HPLC.

Three analytical systems for the HPLC separation of human insulin B chain were studied. In all of them, the stationary phase employed was a μBondapak C18 cartridge (8 mm x 100 mm) with a particle size of 10μ. The mobile phase used in the three systems was an organic solvent and an aqueous buffer A containing reagents which form ion-pairs. Buffer A was composed of 2.5% formic acid and 15mM triethylamine. Table 1 shows the proportion of organic solvent for each system in the equilibrium and elution buffers, as well as the retention times for porcine insulin B chain used as a standard in all systems. In all cases flow rate used was 1 ml/min.

In the systems studied, elution was carried out in two steps: equilibrium and elution (isocratic). This alternative was selected since it

TABLE I

Summary of Solvent Systems and the Retention Times (rt) for Porcine Insulin B chain Utilized as a Standard*.

			(a) μBondapak C18 column	(b) Analytical column with C18 preparative packing	(c)	(d)
			rt(min)	rt(min)	% organic equilibrium	% organic elution
System	I	(Acetonitrile)	17.04	17.04	30%	33%
System	II	(Isopropanol)	18.02	17.60	27%	30%
System	III	(Methanol)	18.30	17.40	60%	70%

* Retention times for porcine insulin B chain subjeted to HPLC in systems I, II and III utilizing (a) a μBondapak C18 and (b) an analytical column with preparative packing. The proportion of organic solvent for each system in the equilibrium and elution buffers is presented in lanes (c) and (d).

is easier to scale up than gradients systems. The equilibrium and the elution phases were chosen so that their composition were similar, thus reducing the time needed to obtain the initial equilibrium.

As it can be seen in fig. 1, we were able to obtain a good separation of insulin B chain from the peptide mixture using the isopropanol solvent system. Similar results were obtained with the acetonitrile and methanol systems, (data not shown).

To scale up the analytical systems previously described, a metallic column (4.6 mm x 150 mm) was packed with 1 gram of Pre-C18 preparative packing using a particle size of 55-105 μ. This experiment allowed the determination of the effect of the particle size on the resolution of the peptide

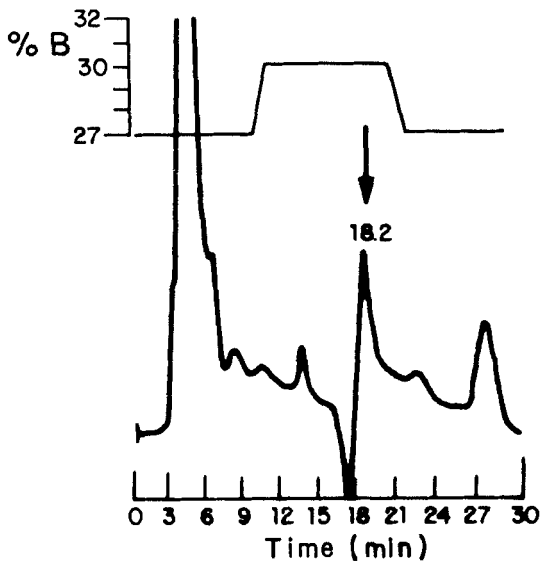

Figure 1. Analytical reverse-phase chromatography of the cyanogen bromide treated inclusion bodies containing the human insulin B chain. A 50 µg peptide sample was passed through a µBondapak C18 column, with a flow rate of 1 ml/min. The position of insulin B chain in the chromatogram is indicated with an arrow. The buffer system used for the purification was: (A) HCOOH 2.5%, TEA 15mM; (B) isopropanol. Equilibrium: 27% B, 10 min; elution: 30% B, 10 min. Detection: 280 nm, 0.05 AUFS.

mixture, and the prediction of the mass load capacity for a larger column with the same packing. The conditions used were the same as for the analytical systems.

As it can be seen in fig. 2 panels I, II and III, the resolution of the peptide mixture diminishes as compared to the analytical system (fig. 1), due to the increment in the packing particle size. However, this decrement in resolution did not affect the objetive of the experiment since retention times for the indicated peaks in fig. 2 are similar to those obtained with porcine insulin B chain used as a standard (see Table I). Polyacrilamide gel electrophoresis of the peaks marked with arrows in fig.

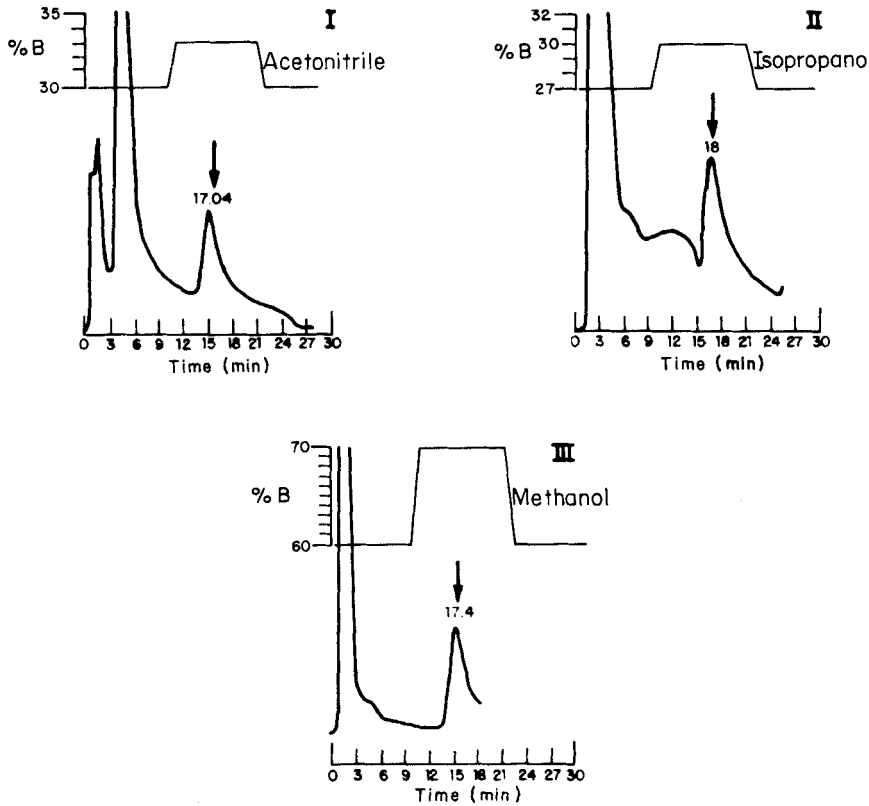

Figure 2. Analytical chromatographic purification of human insulin B chain. A reverse-phase chromatography using an analytical size metallic column with preparative packing of 100 µg of the cyanogen bromide treated peptide mixture containing the insulin B chain polypeptide, is shown in panels I, II and III. Panel I, buffer composition: (A) HCOOH 2.5%, TEA 15mM; (B) acetonitrile; equilibrium: 30% B, 10 min; elution: 33% B, 10 min. Panel II, buffer composition: (A) HCOOH 2.5%, TEA 15mM; (B) isopropanol; equilibrium: 27% B, 10 min; elution: 30% B. 10 min. Panel III, buffer composition (A) HCOOH 2.5%, TEA 15mM; (B) methanol; equilibrium: 60% B, 10 min; elution: 70% B, 10 min. The flow rate used in all systems was 1 ml/min. Detection: 280 nm, 0.02 AUFS. The position of the human insulin B chain in each chromatogram is marked by an arrow.

2 panels I to III revealed a peptide band wich commigrates with the standard (data not shown).

Since the resolution obtained for insulin B chain is similar in all three systems, all of them can be directly scaled up to a preparative level of several grams, once the calculations for mass load capacity and flow rate are performed. On the basis of cost, since this criterion becomes important once the purification process is scaled up, the isopropanol system was selected.

To determine the mass load capacity for a PrepPAK 500 C18 cartridge, maximum load tests were performed in a metallic analytical column (4.6 mm x 150 mm) with 1 g of C18 preparative packing. The maximum protein load determined for this column was 86 mg. After each of the parameters, such as flow rate and time, were defined for the metallic column, the scale-up proportion was calculated using the following equation.

$$Cm = (R_2)^2 / (R_1)^2 \times L_2 / L_1$$

$$Cm = (28.5\ mm) / (2.3\ mm) \times 300\ mm / 150\ mm = 307$$

where Cm is the mass of the sample with known concentration; R_2 is the radius of the larger column and L_2 its length; R_1 is the radius of the analytical column and L_1 its length. This calculation indicated that the amount of material that can be subjected to chromatography in the large column is approximately 300 times the amount of the analytical column, and this, in turn, indicates that 26.4 g of protein can be loaded per run.

Figure 3 panel I shows the chromatogram of a preparative run of 10 g of the bacterial peptide mixture on a PrepPAK 500 C18 cartridge (57 mm x 300 mm), using a flow rate of 100 ml/min in a Prep LC/System 500 A Liquid Chromatrograph. The arrow indicates the position where porcine insulin B chain is resolved. As it can be observed, the profile obtained is similar to the one described for the preparative packed column (fig. 2, panel II). The material from the peak region "D" obtained in this

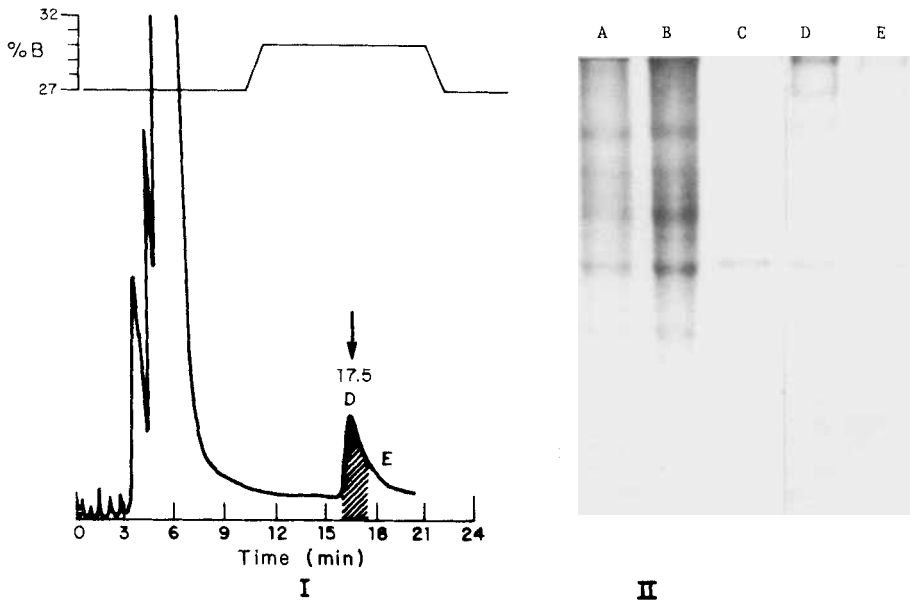

Figure 3. Preparative chromatographic purification of human insulin B chain. I) Preparative reverse-phase chromatography of 10 g of the cyanogen bromide peptide mixture containing the human insulin B chain. A PrePak 500 C18 column utilizing the isopropanol solvent system was used and the flow rate was 100 ml/min. Buffer composition: (A) HCOOH 2.5%, TEA 15mM; (B) isopropanol; equilibrium: 27% B, 10 min: elution: 30%B, 10 min. Detection: 280 nm, 0.1 AUFS using the same detector with a preparative cell. The fractions containing the insulin B chain are marked by letters "D" and "E".
II) PAGE analysis of preparative HPLC purified human insulin B chain. Lane A: 10 µg of cyanogen bromide peptide mixture before purification; Lane B: 20 µg of cyanogen bromide peptide mixture before purification; Lane C: 20 µg of porcine pancreas insulin B chain (standard); Lane D: 5 µg of fraction "D" from the preparative chromatogram; Lane E: 4 µg of fraction "E" from the preparative chromatogram.

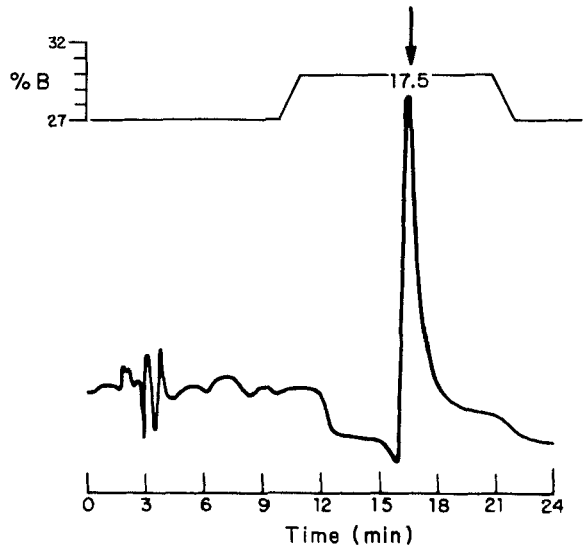

Figure 4. Rechromatography of a sample of material of fraction "D" derived from the preparative scale purification of insulin B chain. A sample of 5 µl from fraction "D" (fig. 3, panel I) was passed through a µBondapak C18 column using the acetonitrile solvent system. Buffer composition: (A) HCOOH 2.5%, TEA 15mM; (B) acetonitrile; equilibrium: 30% B, 10 min; elution: 33% B, 10 min.

purification was collected and a sample was passed through an analytical µBondapak C18 cartridge (8 mm x 100 mm) using the acetonitrile solvent system. Figure 4 shows the chromatograph of this analytical run, where a mayor peak is evident.

As it can be observed in fig. 3, panel II, insulin B chain elutes in a peak that PAGE analysis revealed to be highly enriched for this peptide. Contaminant peptides are still present (fig. 3, panel II, lanes D and E), but in a much smaller proportion than in the unpurified mixture (fig. 3, panel II, lanes A and B). These peptides, originated mainly from the CNBr hydrolysis of the cl carrier protein, however, they do not interfere with the association reaction between the insulin A and B chains, and were eliminated in the HPLC purification of the correctly associated insulin molecules (data not shown) (6,10).

CONCLUSIONS

Starting with 100 g dry weight of bacterial paste containing 20% of its total protein as the cI-insulin B chain hybrid polypeptide, it is possible to obtain, approximately, 1.2 g of human insulin B chain after a four step process: cell sonication, differential centrifugation for the purification of inclusion bodies, cyanogen bromide treatment of the inclusion bodies (6) and the preparative HPLC chromatography described in this communication. This process can be accomplished in four days, with a 62% overall protein recovery.

The human insulin B chain obtained after the preparative HPLC purification has been associated with insulin A chain to produce active human insulin (data not shown) (6,10). This purification method could be further scaled up in order to process a larger amount of biological material, and eventually be used in a process for the large scale production of human insulin.

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REFERENCES

1. Frank, B. H. and Chance, R. Two routes for producing human insulin utilizing recombinant DNA technology. Munch. Med. Wschr. 125 (suppl. 1), 514 (1983).

2. James, L.D. Scaling up bio-product separation with high performance liquid chromatography. Biotechnology 2, 957 (1984).

3. Gail, S. and Charles, M. From R & D to production: designing a chromatographic purification scheme. Biotechnology 5, 239 (1987).

4. Ladrón de Guevara, O., Estrada, G., Antonio, S., Alvarado, X., Güereca, L., Zamudio, F., and Bolivar, F. Identification and isolation of human insulin A and B chains by high-performance liquid chromatograhy. J. Chromat 349, 91 (1985).

5. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680 (1970).

6. Flores, N., de Anda, R., Güereca, L., Cruz, N., Antonio, S., Balbás, P., Bolivar, F. and Valle, F. A new expression vector for the production of fused proteins in Escherichia coli. App. Microbiol. Biotechnol. 25, 267 (1986).

7. Gross, E. The cyanogen bromide reaction. Meth Enzymol. 11, 238 (1967).

8. Cole, R.D. Sulfhytolisis. Meth Enzymol 11, 206 (1967)

9. Goeddel, D.V., Kleid, D.F., Bolivar, F., Heyneker, H.L., Yansura, D.G., Crea, R., Hirose, T., Kraszewski, A., Itakura, K., and Riggs, A.S.D. Expression in Escherichia coli of chemically synthesized genes for human insulin. Proc Natl Acad Sci USA 76, 106 (1979).

10. Balbás, P., de Anda, R., Flores, N., Alvarado, X., Cruz, N., Valle, F. and Bolivar, F. Overproduction of proteins by recombinant DNA: human insulin. Cell funtion and disease (Cañedo, L., Todd, L., Jaz, J. and Parker, L., eds.) Plenum Press, New York, N. Y. 59 (1988).