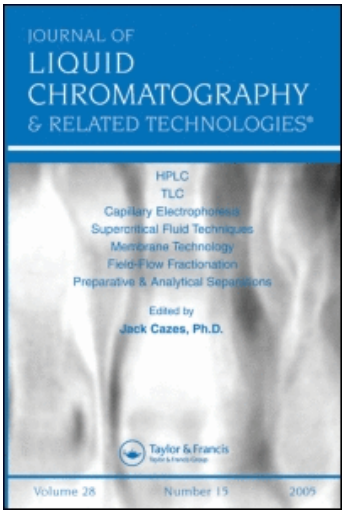

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PREPARATIVE ISOLATION OF RECOMBINANT HUMAN INSULIN-A CHAIN BY ION EXCHANGE CHROMATOGRAPHY

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ABSTRACT

A simple, selective and high capacity process is described for the preparative purification of recombinant human insulin A chain, using ion exchange chromatography. This process was developed considering the particular physicochemical characteristics of this peptide. We have found that the insulin A chain binds strongly to the anionic exchanger Macro Prep 50 Q, which permits the equilibration of the resin to an ionic strength of 0.5 M NaCl. These conditions avoid the adsorption of most contaminant components, thus incrementing the capacity of the support for the insulin A chain. Moreover, the process can be easily automatized and scaled-up.

INTRODUCTION

Proteins produced through genetic engineering technology can be used as active substances, because they are chemically and biologically defined. Recent developments are focused to study and modify proteins to change their specificity in order to design new biological activities for industrial applications (1).

During the last decade, the development of the techniques for gene isolation and expression in different species, specially bacteria, has offered great possibilities, and in principle, today it is possible to produce a great variety of proteins. However, in some cases the expression of heterologous genes presents some inherent factors which may become significative obstacles for the production of proteins, such as the lack of knowledge about the final structure and the final biological activity of the protein produced (2). This aspect has fundamental importance for the development of purification processes, since factors such as temperature, pH, shear forces, oxidation, etc., may affect the final biological activity of the product (3).

Since the purification of recombinant proteins represents usually between 80-90% of the total cost of the process, it is necessary to design efficient methodologies, in order to lower the overall costs (4).

Typical purification processes for recombinant proteins from E. coli, require several chromatographic steps based on different physicochemical properties, such as: hydrophobicity, affinity, ionic exchange and gel permeation (1). As the number of steps increase in a particular purification process, there is also an increment in complexity, associated with higher costs and lower yields. Therefore, it is important to develop purification processes involving the fewest number of steps (5).

We have reported the construction of recombinant hybrid plasmids for the production of human insulin A and B chains in E. coli. These peptides are expressed fused to a fragment of the lambda cI repressor protein, and the in vitro association of the peptide chains yielded active human insulin (6). In order to scale-up these processes, it has been necessary to define the procedures and equipment required to work at larger scales. For these reasons, we have developed chromatographic systems that could be easily scaled-up.

Recently, we have reported the preparative purification of recombinant human insulin B chain, using HPLC (7). A similar procedure can be used for the isolation of the A chain; however,

considering the singularities of this peptide, we have developed an alternative, simpler and economical procedure. The insulin A chain is composed of 21 aminoacid residues, most of them acidic. Between them, there are four cysteine residues that when transformed to S-sulfonates ($A-(SSO^-_3)_4$), there is an increase in the anionic character of the peptide and its solubility; the isoelectric point of this oxidized form is lower than 2 (8). Therefore, in a lightly basic pH, this peptide is strongly negatively charged. Because of these features, it is expected that insulin A chain may be purified using an anionic exchanger in a moderately basic pH.

MATERIALS AND METHODS

Reagents

Formic acid, hydrochloric acid and sodium chloride from Baker. Urea from Merck. Trizma base, cyanogen bromide, sodium tetrationate and sodium sulfite from Sigma. Guanidinium hydrochloride from Pierce. Porcine insulin A chain from Sigma (I-3505) was used as a standard. Macro Prep 50 Q and Biogel P2 from Bio Rad. Dialysis bags with a cutoff of 1000 Da from Spectrum.

Lysis buffer composition: 50 mM Tris, 0.1 mM EDTA and 1 mM ß-mercaptoethanol.

Equipment

Buchi rotaevaporator. Ultrafilter DC 10 l and a hollow fiber cartridge with a filtration surface area of 0.45 and a cutoff value of 100 kDa, from Amicon. Manton Gaulin Homogenizer from Gaulin Corporation. Static centrifuge from Beckman. Columns 1 x 5 cm and 2.5 x 20 cm with adaptable pistons, fraction collector with UV detector and recorder from LKB. Masterflex peristaltic pump from Cole Parmer. For antifoam and precipitated salt removal, a Mont-Inox sieve No. 40 (0.42 mm), was used.

Polyacrylamide gel electrophoresis (PAGE)

Slab gel electrophoresis in the presence of SDS was performed as described by Laemmli (9) for detection of hybrid proteins.

Slab gel electrophoresis without SDS was performed in 19% w/v acrylamide, 1% w/v bis-acrylamide gels for insulin A chain analysis (8).

Hybrid protein and A chain level measurements

The amount of cI-A protein and insulin A chain were determined by scanning polyacrylamide gels with the appropriate samples, using a soft laser scanner densitometer from Biomed Instruments, Inc. model SCR-2D/1D.

Protein determination

The method used was the one reported by Lowry (10).

Growth of recombinant Escherichia coli

The Escherichia coli W3110 strain transformed with the recombinant plasmid pINS-A (unpublished results) was used for the production of the cI-human insulin A chain hybrid protein. The production strain was grown under batch culture conditions using complex medium with the following parameters: temperature 37 °C, pH=7.4, and dissolved oxygen concentration maintained higher than 20%. A cellular concentration of 9 g/l dry weight was obtained, and the hybrid protein reached 9-12% of the total cellular protein.

RESULTS AND DISCUSSION

After fermentation of the E. coli W3110 (pINSA) strain, the cI-A hybrid protein represented between 9-12% of the total cellular protein, as determined by densitometric scanning of SDS-PAGE of total cellular proteins. 18 l of culture containing 9 g/l of E. coli cells dry weight, were sieved to remove the antifoaming agent and precipitated salts and subjected to cross-flow ultrafiltration in order to replace the culture media with water and to concentrate the cells by reducing the volume to 4 liters. Cellular rupture was performed using a Manton Gaulin high pressure homogenizer. Three passes, at 10 °C and 500-600

Kg/cm², were necessary for complete disruption. The recovery and partial purification of the cI-A protein inclusion bodies, was accomplished by 4 cycles of washing the pellet with lysis buffer and centrifugation.

The inclusion bodies were subjected to a cyanogen bromide (cyanogenolysis) reaction (11) to release the A chain from the cI carrier protein. The cI sequence was cleaved to several fragments of different sizes; more protein fragments were also generated from the CNBr action on contaminant proteins. The peptide mixture was further subjected to oxidative sulfitolysis, to protect the sulfhydryl groups of the cysteine residues, transforming them into stable S-sulfonate derivatives A-$(SSO_3^-)_4$ (12). Finally, the reaction mixture was dialyzed against water, in bags with a cutoff value of 1000 Da, to eliminate guanidinium hydrochloride and other salts used in the sulfitolysis reaction. As the ionic strength of the solution decreases, many proteins precipitate; since the insulin A chain is very soluble at ph>5, approximately 90% of it remains in the supernatant at the end of the dialysis. After several fermentations, the amount of A chain obtained varied between 2 to 5%, depending on the efficiency of the induction. Figure 1 shows the protein patterns obtained after these purification steps.

It has been found that the isoelectric point of the A chain as A-$(SSO_3^-)_4$, is lower than 2 (8), and it is also known that the solubility of the tetra S-sulfonate insulin A chain decreases drastically below pH=4 (13). As expected, this peptide is strongly adsorbed to an anion exchanger at neutral or slightly basic pH, when the maximum solubility of the peptide is reached.

A method for the chromatographic isolation of the tetra S-sulfonate A chain, was developed using a macroporous acrylic anion exchanger, Macro Prep 50 Q. The exchanger was equilibrated in a small column (1 x 2 cm), in 50 mM Tris/HCl pH=8 with an ionic conductivity of 1.0 millimhos, and a linear flow rate of 38 cm/hr.

A 5 ml aliquot of peptide sample with a protein concentration of 5.0 mg/ml adjusted to pH=8 with a few drops of NaOH 1 M (final

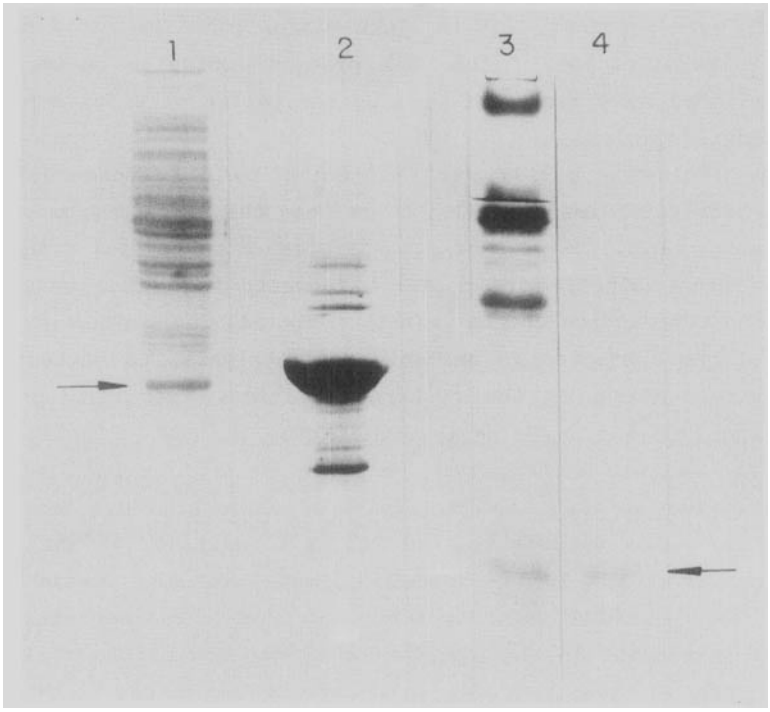

Figure 1.- Denaturing SDS polyacrylamide gel electrophoresis of several protein samples. Lane 1: total proteins from E. coli W3110 (pINSA); Lane 2: protein pattern obtained after purification of inclusion bodies. The arrow in lanes 1 and 2 indicate the position where the hybrid protein migrates; Lane 3: non-denaturing PAGE of peptide mixture obtained after CNBr treatment and sulfitolysis; Lane 4: porcine insulin A chain, used as a standard. The arrow in lanes 3 and 4 indicate the position where the insulin A chain migrates.

conductivity of 2.3 millimhos), was loaded to the column and then washed with six column volumes of equilibration buffer until the baseline was reached. The adsorbed peptides were eluted with ten column volumes of a 0-1 M NaCl linear gradient, followed by five column volumes of 1 M NaCl.

Fractions (3 ml) were collected, and their conductivities determined. Fractions containing NaCl were desalted using Biogel

P2 for PAGE analysis. It was found, see figure 2, that the insulin A chain is the last peptide to elute, at a conductivity of 32-36 millimhos.

From these results, it was evident that it is possible to increase the ionic strength of the buffer, without causing the elution of the A chain. Therefore, a second run was performed using buffer with 0.5 M NaCl, which has a conductivity of 26.5 millimhos, to equilibrate the column. The conductivity of the sample was adjusted by the addition of NaCl. A 5 ml sample was applied with a linear flow rate of 38 cm/hr, followed by eight column volumes of equilibration buffer and a 0.5 - 1.0 M NaCl linear gradient was applied to elute proteins. As can be seen in figure 3, the amount of adsorbed protein was reduced considerably, and the predominant component adsorbed in these conditions was insulin A chain. After elution, an electrophoretic analysis revealed a purity of at least 95% for this peptide. The remaining 5% represented mainly the desamido form of the A chain (14).

Since the insulin A chain is the unique component adsorbed to the anion exchange resin in conditions of 26.5 millimhos (0.5 M NaCl), it was concluded that instead of a gradient, a single elution step could be applied, to elute the A chain as a pure peptide.

Therefore, a 5 ml sample was loaded to the column under the previously described conditions, and elution was done with five column volumes of buffer containing 1 M NaCl (48 millimhos). The electrophoretic analysis of the fractions from this chromatography, shown in figure 4, clearly demonstrated a high degree of purity for the resolved insulin A chain.

To determine the maximum load capacity for the exchanger, 1000 ml of the peptide sample, were applied to a column (2.5 x 15 cm), containing 75 ml of packing, using a linear flow rate of 51 cm/h. Collected fractions were analyzed by PAGE, to determine when the insulin A chain was no longer retained. As seen in figure 5, under this high ionic strength conditions (26.5 millimhos), the bed can be loaded with 920 ml of sample, without

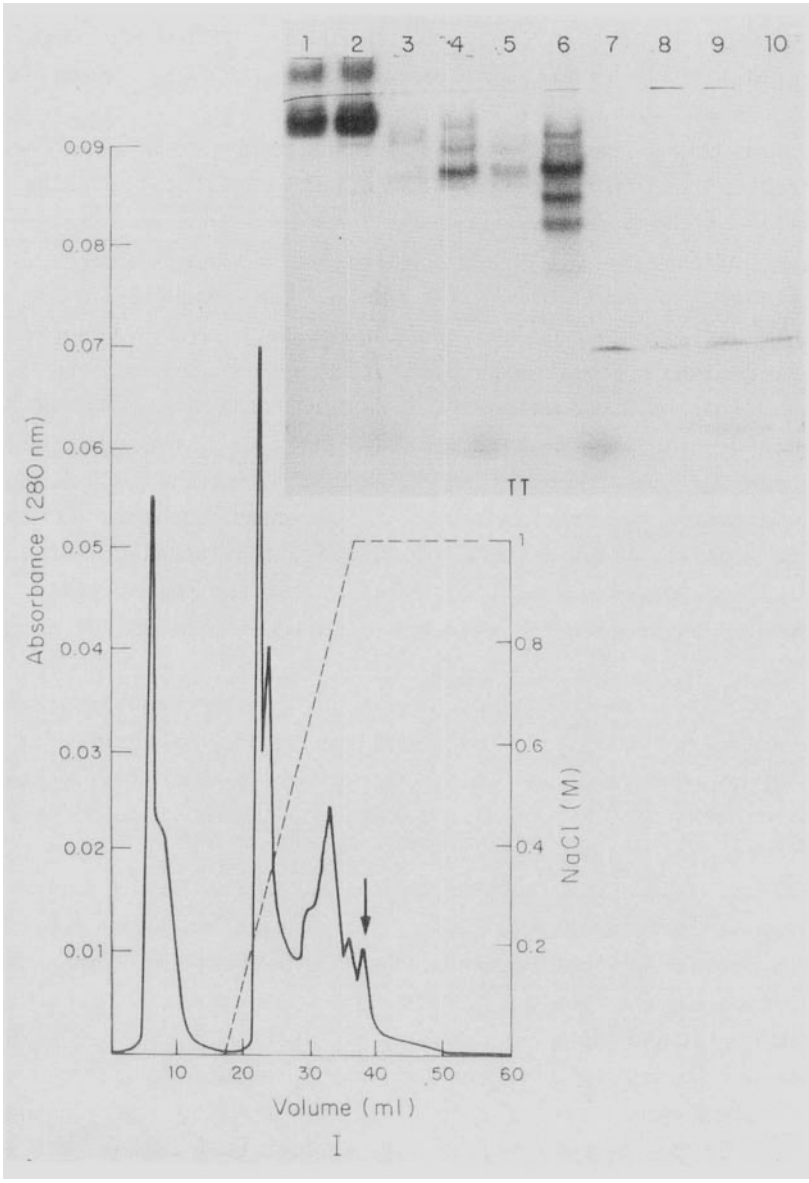

Figure 2.- I.- Chromatographic purification of 5 ml of a cyanogen bromide treated peptide sample in a column packed with 2 ml of preparative packing Macro-Prep 50 Q using a linear flow rate of 38 cm/h. Gradient: 0-1 M NaCl in a total of ten column volumes. The arrow indicates the peak where the insulin A chain elutes. II.- PAGE using 19% acrylamide crosslinked with 1% bis-acrylamide. Lanes 1-6 fractions collected before the A chain elution; lane 7 porcine insulin A chain as standard; lanes 8-10 represent the last fractions from the last peak.

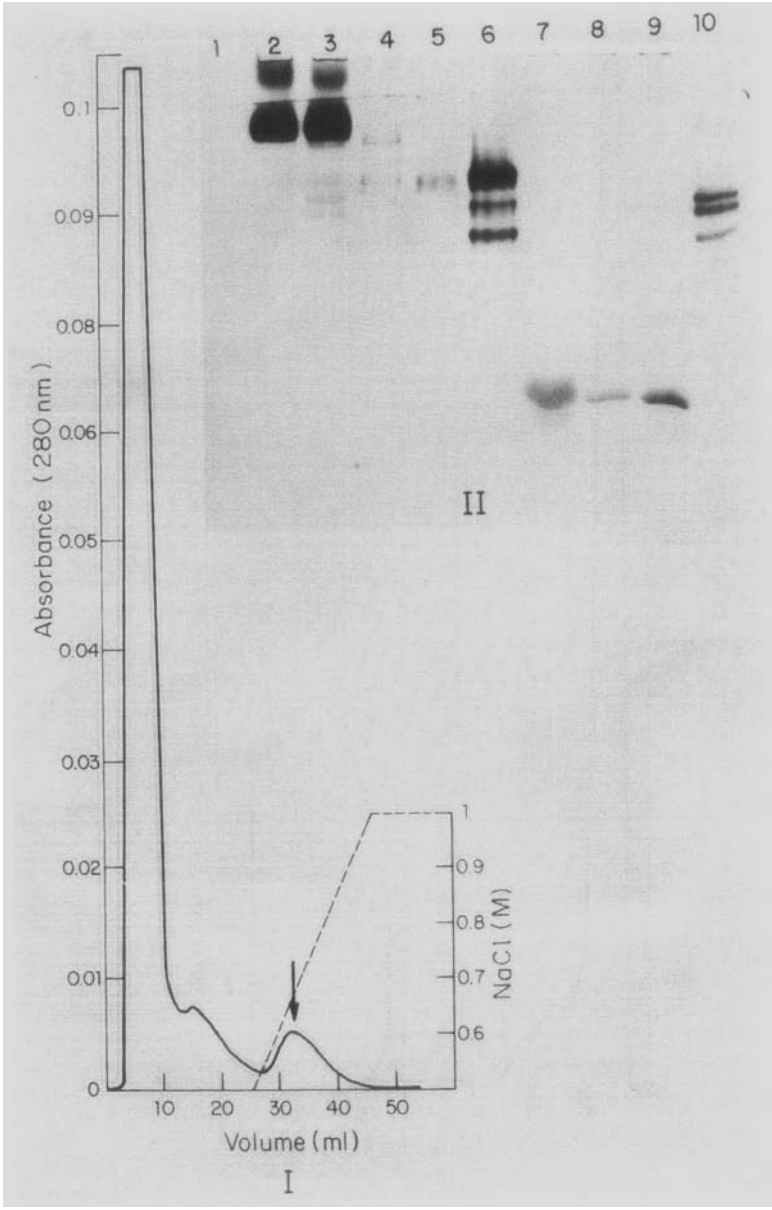

Figure 3.- I.- Chromatographic purification of 5 ml of a cyanogen bromide treated peptide sample in a column packed with 2 ml of preparative packing Macro-Prep 50 Q using a linear flow rate of 38 cm/h, equilibrated with a buffer composed of 50 mM Tris/HCl pH=8 + 0.5 M of NaCl. The arrow indicates the peak were the A chain elutes. Gradient: 0.5-1 M NaCl in a total of ten column volumes. II.- Non-denaturing PAGE. Lanes 1-3, fractions of non adsorbed proteins; lanes 4-6, fractions eluted with equilibration buffer; lane 7, porcine insulin A chain as a standard; lanes 8 and 9, fractions of the peak eluted between 30 and 40 ml; lane 10, initial sample.

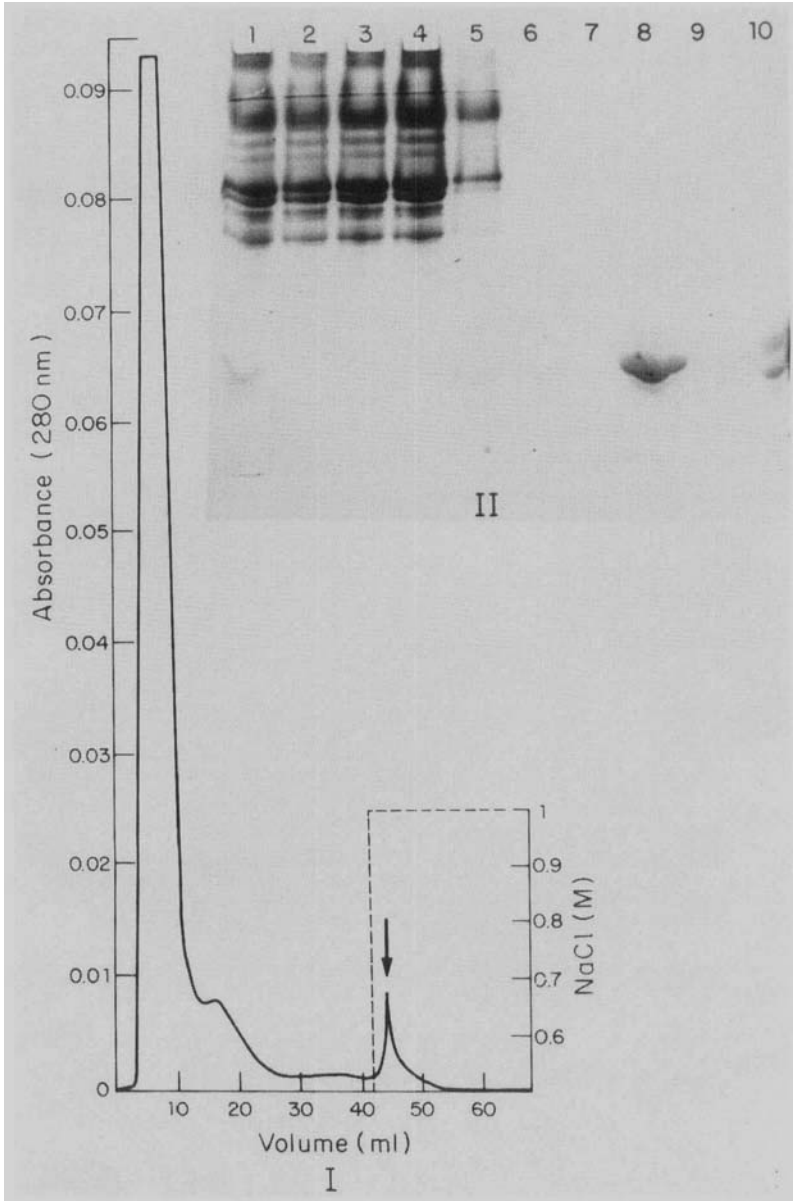

Figure 4.- I.- Chromatographic purification of 5 ml of a cyanogen bromide treated peptide sample in a column packed with 2 ml of preparative packing Macro-Prep 50 Q using a linear flow rate of 38 cm/h, equilibrated with a buffer composed of 50 mM Tris/HCl pH=8 + 0.5 M of NaCl. Elution was performed in a single step with equilibration buffer + 1 M of NaCl. The arrow indicates the fraction where the insulin A chain elutes. II.- Non-denaturing PAGE. Lane 1, initial sample, lanes 2-4, fractions of non-adsorbed protein; lanes 5-7, fractions of proteins eluted with equilibration buffer; lane 8, sample of the principal peak eluted; lane 9, fraction after elution of the principal peak; lane 10, porcine insulin A chain as a standard.

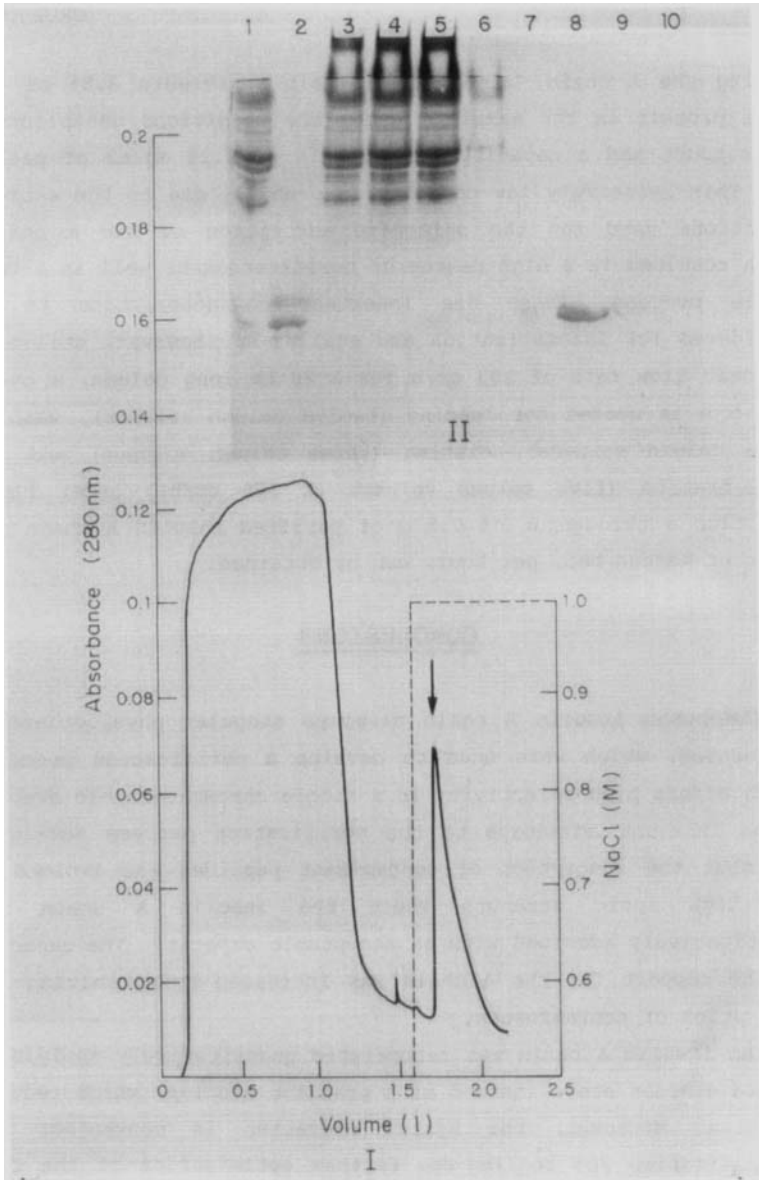

Figure 5.- I.- Chromatographic purification of 1000 ml of the cyanogen bromide treated peptide mixture (5 mg/ml) in a 2.5 X 20 cm column, packed with 75 ml of Macro-Prep 50 Q preparative packing, using a linear flow rate of 51 cm/h. Elution was carried out in the same conditions as indicated in figure 4. The arrow indicates the fraction were the insulin A chain elutes. II.- Non-denaturing PAGE. Lane 1, initial sample; lane 2, porcine insulin A chain used as a standard; lanes 3-5, fractions of non-adsorbed proteins; lane 6-7, proteins eluted with equilibration buffer, lanes 8-10; fractions of the principal peak.

eluting the A chain. Since the A chain represents 3.5% of the total protein in the sample, under the conditions established, the support had a capacity for A chain of 2.14 mg/ml of packed bed. This relatively low capacity is probably due to the extreme conditions used for the selective adsorption of the A chain, which resulted in a high degree of purification as well as a very simple process. These are important characteristics to be considered for automatization and scaling-up. However, utilizing a linear flow rate of 100 cm/h for a 20 cm long column, a cycle of 4.5 h is needed for loading (twelve column volumes), washing (five column volumes), eluting (three column volumes) and re-equilibration (five column volumes at 200 cm/h); under these condition a throughput of 0.5 g of purified insulin A chain per liter of packed bed, per hour, can be obtained.

CONCLUSIONS

The human insulin A chain displays singular physicochemical properties, which were used to develop a purification process, which offers high selectivity in a single chromatographic step.

An inherent advantage to the purification process developed was that the adsorption of contaminant peptides was avoided at the high ionic strength where the insulin A chain was quantitatively adsorbed with an acceptable capacity. The capacity of the support for the A chain was increased by minimizing the adsorption of contaminants.

The insulin A chain was recuperated quantitatively applying a single elution step, instead of a gradient elution, which reduced dilution. Moreover, the system operation is convenient for automatization and scaling-up. Further optimization of the flow rate at pilot-scale operation is needed to maximize throughput.

The purity of the human insulin A chain (95%) obtained by this method has been found adequate for chemical association with recombinant human B chain to yield human insulin with the expected efficiency (data not shown) (6,7).

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