Chromatographic Separation of Aminoethylated Insulin A and B Chains*

R. E. Humbel, R. Derron, and P. Neumann

ABSTRACT: Reduced and aminoethylated insulin A and B chains have the same net charges at neutral pH. Their separation by ion-exchange chromatography is therefore not easily achieved. A simple and reproducible method for the separation of reduced and aminoethylated insulin chains is described using chromatography on IRC-50 resin with linear gradients of acetic or formic acid from 5 (in water) to 100%. The separated chains are free from cross-contamination, as judged by amino acid analyses. The procedure may be applied equally well to the separation of oxidized insulin chains. Aminoethylated fish insulin chains of equal net charges at acid pH could be successfully resolved by this procedure.

The introduction of additional sites for tryptic digestion of proteins by prior aminoethylation of cysteinyl residues (Lindley, 1956; Raftery and Cole, 1963) has become a valuable tool in the elucidation of the amino acid sequence of proteins. In the case of insulin, reduction of the disulfide bridges and alkylation of the resulting SH groups by ethyleneimine affords yet another means of preparing A- and B-chain derivatives. However, the separation of the reduced and aminoethylated beef insulin A and B chains is not easily achieved because their net charges at neutral pH are equal. On the other hand, *S*-sulfonated and reduced carboxymethylated insulin chains have large charge differences and can, therefore, easily be separated by ion-exchange chromatography (Bailey and Cole, 1959; Humbel and Crestfield, 1965).

In order to prepare reduced and aminoethylated insulin B chain, Raftery and Cole (1963, 1966) first separated the chains in the *S*-sulfonate form which were then separately reduced and aminoethylated. Baldesten (1966) reported on the separation of reduced and aminoethylated insulin chains by gel filtration on Sephadex G-50. This latter procedure, however, does not give a complete separation of the two chains.

We have investigated the possibility of separating a mixture of reduced and aminoethylated A and B chains by modifying the procedure of Crestfield *et al.* (1963) for isolating reduced carboxymethylated insulin B chain on IRC-50. This paper presents data on the successful separation of beef and fish insulin reduced and aminoethylated chains using stepwise or gradient elution from IRC-50 with formic or acetic acid.

Materials and Methods

Crystalline beef insulin (IUPAC) (lot no. 26918) was obtained from British Drug Houses. Another lot of crystalline beef insulin was a gift of the Hoechst Co. A mixture of bonito and tuna insulin was obtained from the Shimizu Seiyaku Co., Japan. By chromatography on CM-cellulose by a previously described procedure (Humbel and Crestfield, 1965) a pure preparation was obtained whose amino acid composition is almost identical (P. Neumann, unpublished data) with the one of tuna insulin II (K. Satake and M. Tanaka, personal communication). It will henceforth be referred to as tuna insulin II. IRC-50 resin (−400 mesh) was obtained from Bio-Rad. All reagents used were of analytical grade.

Crystalline zinc insulin (120 mg) was dissolved in 16 ml of 8 M urea, containing 0.35 M Tris-HCl (pH 8.6) and 6.5 mM EDTA. Mercaptoethanol (final concentration, 0.13 M) was added under nitrogen. After 4 hr at room temperature, 0.8 ml of ethylenimine (final concentration, 0.92 M) was added. After another 30 min, the mixture was acidified with 10 ml of 1 M acetic acid, dialyzed in $^{18}/_{32}$ Visking tubing against distilled water for 20 hr, and then lyophilized; yield, 103 mg.

For the separation of 5–30 mg of reduced and aminoethylated chains, IRC-50 resin was used in a column of 0.9 × 30 cm. The column was freshly poured before each run and equilibrated with 5% (v/v) aqueous formic or acetic acid. The sample was applied in 1 ml of 5% formic or acetic acid. Elution was carried out by a linear gradient of formic or acetic acid (from 5 to 100%) with a flow rate of 18 ml/hr, the total volume in mixing and reservoir chambers being 132 ml. The gradient-producing device has to be made of acid-resistant material. Perspex is dissolved by concentrations of carboxylic acids greater than about 50%. The effluent was monitored by its absorbancy at 280 mμ with a LKB Uvicord II. Appropriate fractions were pooled, concentrated on a flash evaporator, diluted with water, and lyophilized.

For separations on a larger scale, 100 mg of reduced and aminoethylated insulin was dissolved in 4 ml of

* From the Institute of Biochemistry, University of Zürich, Zürich, Switzerland. *Received October 9, 1967.* This work was supported in part by a grant from the Schweizerische Nationalfonds (3900).

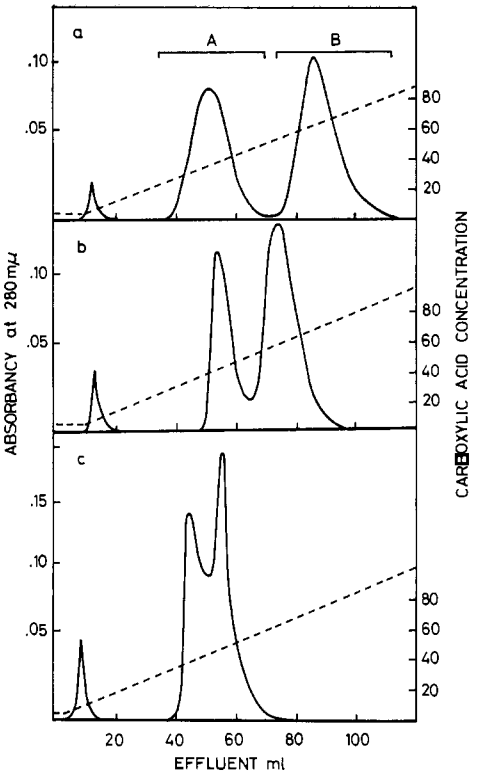

FIGURE 1: Separation of beef reduced and aminoethylated insulin A and B chains on IRC-50. Column, 0.9 × 30 cm; load, 30 mg of reduced and aminoethylated insulin. Gradient: (a) 5–98% formic acid, (b) 5–100% acetic acid, and (c) 5–99% propionic acid.

25% acetic acid and applied on a 2.5 × 10 cm column of IRC-50 in 25% acetic acid. Reduced and aminoethylated A chain was washed through with 200 ml of 25% acetic acid, whereas reduced and aminoethylated B chain could be eluted with 100 ml of 60% acetic acid. The two separate fractions were diluted with water and lyophilized; yield of reduced and aminoethylated A chain, 35 mg; yield of reduced and aminoethylated B chain, 59 mg.

Amino acid analyses of acid hydrolysates were performed on a Beckman–Spinco amino acid analyzer 120B (Spackman *et al.*, 1958). Oxidation of insulin was carried out according to the procedure of Sanger (1949), sulfitolysis according to Bailey and Cole (1959), and reduction and carboxymethylation according to Crestfield *et al.* (1963).

Results

Figure 1 shows the elution pattern of 30 mg of reduced and aminoethylated insulin from IRC-50 with linear gradients of formic, acetic, and propionic acids at room temperature. The peak of reduced and aminoethylated A chain emerges from the column at an influent acid concentration of 36, 41, and 38% for formic, acetic, and propionic acids, respectively, the

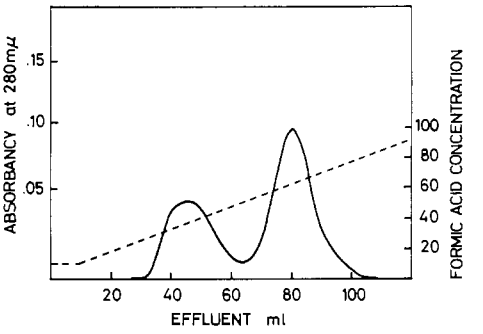

FIGURE 2: Separation of fish reduced and aminoethylated insulin A and B chains on IRC-50. Column, 0.9 × 30 cm; load, 30 mg of reduced and aminoethylated insulin; and gradient, 5–98% formic acid.

peak of reduced and aminoethylated B chain at 64, 59, and 47%, respectively. It can be clearly seen that formic acid yields slightly better separation than acetic acid, whereas separation in propionic acid is unsatisfactory. Reduced and aminoethylated chains of tuna insulin II which has two additional basic residues in its A chain (P. Neumann, unpublished data) are somewhat less well resolved into component chains (Figure 2). By the same procedure, oxidized beef insulin chains are easily separated (Figure 3). *S*-Sulfonated and reduced carboxymethylated insulin chains are only sparingly soluble in 5% carboxylic acid. When the soluble portion of carboxymethylated insulin chains was applied to the column, pure carboxymethylated B chain emerged at an influent formic acid concentration of 68%. Dissolving carboxymethylated insulin chains in 50% acetic acid and eluting with a gradient from 50 to 100% acetic acid gave no satisfactory separation. Similarly, chromatography of *S*-sulfonated chains was unsuccessful.

In addition to insulin derivatives, unmodified insulin could also be chromatographed by this procedure. The

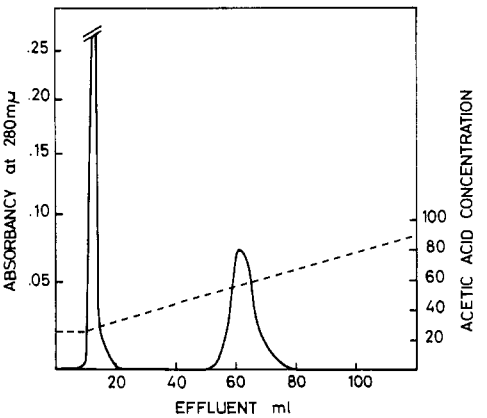

FIGURE 3: Separation of oxidized beef insulin chains on IRC-50. Column, 0.9 × 30 cm; load, 30 mg of oxidized insulin; and gradient, 5–98% formic acid.

peak of insulin was eluted at an influent acetic acid concentration of 47%.

Table I shows the influence of temperature on the elution profile. It can be seen that the elution volume is inversely related to the temperature. The flow rate could be changed from 9 to 36 ml/hr with no gross change in chromatographic behavior. In the larger scale preparation using stepwise elution, 25% acetic acid is a sufficient concentration to wash through reduced and aminoethylated A chain in the case of beef insulin. For tuna insulin II reduced and aminoethylated A chain, an initial concentration of 35% acetic acid has to be used. The purity of the chains obtained by either gradient or stepwise elution was checked by amino acid analyses of the hydrolyzed fractions. Cross-contamination of isolated reduced and aminoethylated chains was found to be never in excess of 3% in the case of beef insulin. In tuna insulin chains, cross-contamination was sometimes somewhat higher. Rechromatography of the isolated fractions caused in all cases chain preparations with no cross-contamination detectable by amino acid analysis.

In the course of sequence studies on tuna insulin II (P. Neumann, unpublished data), reduced and aminoethylated chains were prepared by the method described here. Analyses of tryptic peptides gave no evidence of peptide-bond hydrolysis due to exposure to formic acid.

TABLE I: Effect of Temperature on Elution of Reduced and Aminoethylated Insulin Chains from IRC-50 (column dimensions, 0.9 × 30 cm) with a Linear Gradient of Formic Acid from 5 to 98%.

	Formic Acid Concn in %[a]		
RAE Chain	4°	24°	44°
RAE A	50	36	31
RAE B	74	64	55

[a] The figures give the concentrations of influent formic acid at the emergence of the peak fraction from the column. RAE, reduced and aminoethylated.

Discussion

For the preparation of reduced carboxymethylated insulin B chain, Crestfield *et al.* (1963) used Dixon's (1959) desalting procedure on IRC-50. A chain was washed through together with urea and reagents in 5% acetic acid, and B chain could be eluted with 50% acetic acid. Heinrikson *et al.* (1965) further refined this procedure for the separation of tryptic peptides of ribonuclease by applying linear gradients of acetic acid.

Since a simple and satisfactory procedure for the separation of reduced and aminoethylated A and B chains was not available, a systematic reinvestigation of the IRC-50 procedure was undertaken. The method described here has the practical advantage of being simple, reproducible, and of yielding isolated reduced and aminoethylated A and B chains free from cross-contamination. Eluted fractions can be directly concentrated on a flash evaporator and lyophilized without the need of a prior desalting step.

Acknowledgments

We gratefully acknowledge the skillful assistance of Mrs. A. Gacond. One of us (R. E. H.) is indebted to the Hoechst Co., Frankfurt, for financial support.

References

Bailey, J. C., and Cole, R. D. (1959), *J. Biol. Chem. 234*, 1733.

Baldesten, A. (1966), *Acta Chem. Scand. 20*, 270.

Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238*, 622.

Dixon, H. B. F. (1959), *Biochim. Biophys. Acta 34*, 251.

Heinrikson, R. L., Stein, W. H., Crestfield, A. M., and Moore, S. (1965), *J. Biol. Chem. 240*, 2921.

Humbel, R. E., and Crestfield, A. M. (1965), *Biochemistry 4*, 1044.

Lindley, H. (1956), *Nature 178*, 647.

Raftery, M. A., and Cole, R. D. (1963), *Biochem. Biophys. Res. Commun. 10*, 467.

Raftery, M. A., and Cole, R. D. (1966), *J. Biol. Chem. 241*, 3457.

Sanger, F. (1949), *Biochem. J. 44*, 126.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.