ANION EXCHANGE CHROMATOGRAPHY OF OXIDIZED INSULIN PEPTIDES

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The separation of the A and B chains of performic acid-oxidized insulin is frequently desired for various biochemical investigations, such as the determination of bond specificities of newly-isolated proteinases[1–4]. An interest in determining the bonds hydrolyzed by some proteinases we have isolated prompted a search for a convenient procedure to separate the oxidized A and B chains (glycyl and phenylalanyl peptides, respectively) in amounts sufficient to permit their use as substrates. It was desirable that the procedure obviate such specialized equipment as the countercurrent distribution train required by the procedure of CRAIG, KONIGSBERG AND KING[5]; it was mandatory that it produce peptides of satisfactory purity and that it be quick and reproducible, inasmuch as production of the peptides was to be routine. We describe herein a convenient chromatographic procedure for the separation of A and B chains of oxidized insulin in a highly pure, salt-free state.

EXPERIMENTAL

Materials

Insulin, six-times crystalline, was obtained from Boots Pure Drug Co., Ltd., Nottingham, England. DEAE-Sephadex A-25, fine particle size, capacity 2.9 mequiv./g, was purchased from Pharmacia Fine Chemicals, Inc. Performic acid was prepared from 97–100 % formic acid (Matheson, Coleman and Bell), and Merck Reagent Grade Superoxol (approximately 33 % H_2O_2). Tris-(hydroxymethyl)-aminomethane was purchased from Mann Research Laboratories. The 6 *N* hydrochloric acid used for hydrolysis was prepared either by diluting freshly opened Reagent Grade HCl (Fisher Scientific Co.) or by distillation in glass. All other chemicals used were Reagent or Analytical grade.

Oxidation of insulin

The method of CRAIG *et al.*[5] was used without modification for the performic acid oxidation of insulin at 0°. The oxidized product was lyophilized and stored at —15°.

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Preparation of DEAE-Sephadex columns

A number of experiments were performed using both DEAE-cellulose and DEAE-Sephadex columns with a variety of buffer systems. Although good separations were obtained with both types of exchangers, most of the experiments were performed with DEAE-Sephadex. Therefore, only the procedure using DEAE-Sephadex is described herein.

DEAE-Sephadex was allowed to swell in an excess of distilled water; the water was removed by vacuum filtration and the filter cake was suspended in 0.5 *N* HCl. The HCl was removed immediately by vacuum filtration, and the DEAE-Sephadex was washed several times in water. The filter cake was suspended in 0.5 *N* NaOH and allowed to stand for about 15 min. The suspension was filtered, washed several times with water and finally washed several times with the initial buffer. The exchanger was then packed into a chromatographic tube of a size selected on the basis of the sample size. A column of 1.2 × 21 cm was poured for samples of oxidized insulin of 25–50 mg; for samples of 50–250 mg, a column of 2 × 27 cm was used.

Chromatographic procedures

A sample of the desired amount of oxidized, lyophilized insulin was dissolved in distilled water by the dropwise addition of 0.5 *N* base; NaOH was used when the sample was to be eluted with NaCl, and NH_4OH was used when an ammonium formate gradient was to be used. The sample was applied to the column in the minimum volume.

Elution was accomplished with gradients of either ammonium formate or NaCl. For elution with ammonium formate, a linear gradient was used, with 300 ml of 0.01 *M* ammonium formate (adjusted to pH 9.5 with NH_4OH) in the first chamber of an Autograd (Technicon Chromatography Corp.), and with 300 ml of 1.0 *M* ammonium formate (pH 9.5) in the second chamber. For elution with NaCl, a compound gradient was obtained by loading the Autograd as follows: Chamber 1, 0.005 *M* Tris·HCl; Chambers 2 and 3, 0.032 *M* Tris·HCl, 0.3 *M* in NaCl; Chambers 4 and 5, 0.041 *M* Tris·HCl, 0.4 *M* in NaCl; Chamber 6, 0.050 *M* Tris·HCl, 0.5 *M* in NaCl. The Tris buffer was pH 8.0 in all chambers, each of which contained 75 ml. Flow rates were approximately 1 ml/min; column effluents were monitored at 280 mμ with an Ultraviolet Absorption Meter (Gilson Medical Electronics) and a "Rectiriter" recorder (Texas Instruments, Inc.). Fractions (10 ml) were collected in a G.M.E. fraction collector. In some experiments the positions of the fractions were located by reading the effluent in a Beckman DU Spectrophotometer at 280 mμ, or by the alkaline hydrolysis–ninhydrin method of HIRS *et al.*[6].

Desalting

Samples eluted by the ammonium formate were desalted by sublimation in a freeze-drying apparatus whose condenser was chilled in a bath of solid CO_2 and methylcellosolve. A pressure of 0.05 mm or less was maintained, and heat from infrared lamps was applied to the flasks containing the samples to aid in sublimation of the volatile salt.

Fractions from the NaCl elution were desalted by passage through a 1.2 × 30 cm column of Sephadex G-25.

Amino acid analyses

The samples of fractions to be analyzed for amino acids were hydrolyzed *in vacuo* with 6 *N* HCl by the method of MOORE AND STEIN[7]. The de-aeration procedure recommended by these authors was used. After hydrolysis for 22 or 96 h, the excess HCl was removed by repeated evaporation in a rotary evaporator, the samples were taken to dryness, then dissolved in 1 % HCl for application to the column of the automated amino acid analyzer.

A Technicon Amino Acid Analyzer with a 130 cm column of Chromobeads B was used for amino acid analysis. Standards were run before and after each series of unknowns, and norleucine was used as an internal standard with each unknown sample.

RESULTS

Separation of the oxidized A and B chains of insulin was readily achieved with either the ammonium formate or the Tris–NaCl gradients. The former, however, was simple and convenient, and was more frequently used. Fig. 1 shows a chromatogram in which the ammonium formate gradient was applied. The chromatograms were quite reproducible, with the B chain (Fraction II) emerging at approximately 0.4 *M* ammonium formate, and the A chain (Fraction III) at approximately 0.6 *M*. The identity of Fraction I was not extensively investigated, but it appeared to consist of undefined mixtures of the oxidized A and B chains. A single experiment revealed the presence of all amino acids of both chains, but the molar ratios of amino acids in

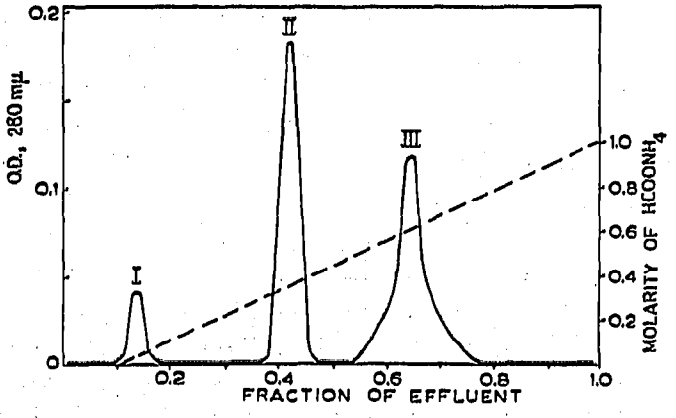

Fig. 1. Chromatography of oxidized insulin on DEAE-Sephadex, with elution by ammonium formate gradient. Fraction I is an unidentified impurity, Fraction II is the B chain, and Fraction III is the A chain.

the hydrolysate suggested that there was relatively more B chain than A chain. The material from Fraction I in this experiment also contained a non-protein component which was not identifiable by spectrophotometric analysis. The absence of methionine from hydrolysates of the preparations ruled out the possibility that glucagon contributed to the impurity. CRESTFIELD, MOORE AND STEIN[8] also found an impurity in the separation of reduced, S-carboxymethylated insulin peptides by gel filtration.

The shape of the peak representing the B chain suggested the presence of a

single component. This is borne out by the amino acid analyses shown in Table I, whose values are in satisfactory agreement with the known composition of this peptide, and with the values reported from the B chain by CRAIG *et al.*[5], who separated the peptides by counter-current distribution. Isoleucine, the "marker" amino acid for A chain, was barely detectable.

TABLE I

AMINO ACID COMPOSITION OF OXIDIZED INSULIN B CHAIN (PHENYLALANYL PEPTIDE)*

Amino acid	*μmoles found*	*No. of residues per mole*	
		Found	*Theoretical*
Cysteic acid	0.053**	1.83**	2
Cystine	0.000	0.00	0
Aspartic acid	0.031	1.08	1
Threonine	0.033	1.14	1
Serine	0.027	0.93	1
Glutamic acid	0.079	2.72	3
Proline	0.033	1.14	1
Glycine	0.088	3.01	3
Alanine	0.065	2.22	2
Valine	0.088	3.01	3
Isoleucine	0.002	0.07	0
Leucine	0.114	3.91	4
Tyrosine	0.054	1.86	2
Phenylalanine	0.088	3.01	3
Lysine	0.034	1.15	1
Histidine	0.056	1.93	2
Arginine	0.028	0.96	1
Ammonia	0.1725	5.94	2

* A 22-h hydrolysate was prepared as described in the text.

** Corrected by dividing by 0.94 (ref. 9). The values for other amino acids have not been corrected for destruction during hydrolysis.

Amino acid analyses of Fraction III (Fig. 1) normally revealed the presence of small amounts of amino acids known not to be in the A chain, but present in the B chain (Table II). The amount of contamination was obviously low, but it appeared desirable to attempt its removal. Consequently, the A chain was rechromatographed on DEAE-Sephadex with the same gradient used in the initial separation of the two

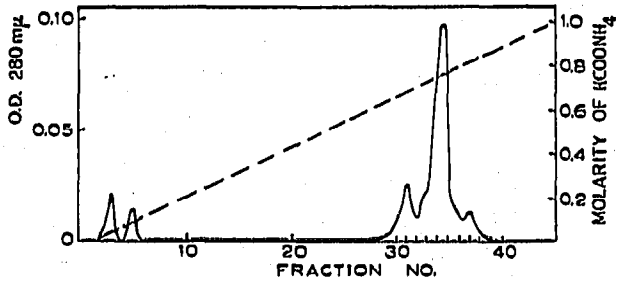

Fig. 2. Rechromatography of oxidized A chain of insulin on DEAE-Sephadex. The sample taken for amino acid analysis consisted of Fractions 34–36.

TABLE II

AMINO ACID COMPOSITION OF OXIDIZED INSULIN A CHAIN (GLYCYL PEPTIDE)*

Amino acid	*μmoles found*	*No. of residues per mole*	
		Found	*Theoretical*
Cysteic acid	0.234**	3.75**	4
Cystine	0.000	0.00	0
Aspartic acid	0.134	2.15	2
Threonine	trace	trace	0
Serine	0.117	1.88	2
Glutamic acid	0.241	3.86	4
Proline	0.000	0.00	0
Glycine	0.071	1.13	1
Alanine	0.071	1.14	1
Valine	0.116	1.85	2
Isoleucine	0.044	0.70	1
Leucine	0.136	2.17	2
Tyrosine	0.120	1.92	2
Phenylalanine	0.004	0.06	0
Lysine	0.004	0.06	0
Histidine	0.001	0.02	0
Arginine	trace	trace	0
Ammonia	0.384	6.16	4

* A 22-h hydrolysate was prepared as described in the text.
** Corrected by dividing by 0.94 (ref. 9). The values for other amino acids have not been corrected for destruction during hydrolysis.

TABLE III

AMINO ACID COMPOSITION OF RECHROMATOGRAPHED A CHAIN OF OXIDIZED INSULIN*

Amino acid	*μmoles found*	*No. of residues per mole*	
		Found	*Theoretical*
Cysteic acid	0.176**	4.15**	4
Cystine	0.000	0.00	0
Aspartic acid	0.093	2.19	2
Threonine	0.000	0.00	0
Serine	0.083	1.96	2
Glutamic acid	0.165	3.89	4
Proline	0.000	0.00	0
Glycine	0.047	1.10	1
Alanine	0.048	1.12	1
Valine	0.078	1.83	2
Isoleucine	0.031	0.72	1
Leucine	0.092	2.16	2
Tyrosine	0.084	1.98	2
Phenylalanine	0.000	0.00	0
Lysine	0.000	0.00	0
Histidine	0.000	0.00	0
Arginine	0.000	0.00	0
Ammonia	0.229	5.40	4

* A 22-h hydrolysate was prepared as described in the text.
** Corrected by dividing by 0.94 (ref. 9). The values for other amino acids have not been corrected for destruction during hydrolysis.

peptides. Fig. 2 shows the rechromatography of the A chain, and in Table III are the results of amino acid analyses on the rechromatographed peptide. Those amino acids distinctive to the B chain (threonine, phenylalanine, proline, and the basic amino acids) were not detected, and the values for the other residues are close to the theoretical values for the A chain. The slightly low values for valine and isoleucine in Tables II and III can probably be ascribed to the resistance of the isoleucyl–valyl bond to acid hydrolysis[8].

The higher-than-theoretical values for ammonia shown in Tables I–III are doubtless an indication that the sublimation procedure did not completely remove the ammonium formate present in the effluent. Nevertheless, the ammonia values are sufficiently low to show the effectiveness of the desalting for most purposes. Desalting the effluent from NaCl elution by gel filtration on Sephadex G-25 was not completely effective in our hands. The procedure was not pursued extensively, however, and there is no reason to assume that it cannot be made satisfactory. Furthermore, removal of NaCl by ion retardation resins[10] should be feasible.

Inasmuch as our purpose was to use the peptides as substrates for proteolytic enzymes, we were primarily concerned with the purity of the separated chains rather than with yields; therefore, the fractions were cut quite sharply by retaining only a few tubes near the centers of the peaks for analyses and for use as substrates. In one experiment the total recovery of all fractions from the column was 77 %. Failure to achieve higher recovery in this experiment can be ascribed largely to mechanical losses incurred in removing the dried material from the lyophilizer flasks.

Preliminary experiments using the bead forms of DEAE-Sephadex A-25 indicated that the gradient would need to be altered somewhat in order to achieve separations comparable to those obtained with the block-polymerized, fine particle size material used in the experiments described herein.

DISCUSSION

The separation of the two peptides resulting from the performic acid oxidation of insulin has been effected by several non-chromatographic procedures, involving precipitation[11,12], counter-current distribution[5], and zone electrophoresis[13]. Previously described chromatographic procedures have been based on the use of a cation exchange resin (Dowex 50) to separate performic acid-oxidized insulin peptides[14] and S-sulfonate peptides derived from treatment of insulin with sulfite[15,16]. The use of cation exchange resins permits the glycyl peptide to pass through the column unretarded[14–16]. Our rationale in selecting anion exchange materials for the separation of the oxidized insulin peptides was based on the difference in the number of sulfonic acid residues in the two chains, to permit the retention of both peptides on the column, from which they could be selectively eluted by a gradient.

We recently became aware of a short communication by FITTKAU[17], published while our work was in progress, in which he reported the separation of performic acid-oxidized insulin peptides by chromatography on 1 × 2 cm columns of DEAE-Sephadex. The peptides were identified by zone electrophoresis and end group analysis, but no analytical values for amino acids in the products were given. FITTKAU's procedure involved a step-wise elution, using 20 % formic acid to remove the B chain, and 1 *N* HCl to elute the A chain. Our procedure appears to have an advantage over

the step-wise elution of the peptides, as the latter would not be expected to separate such impurities as that represented by Fraction I (Fig. 1). Moreover, the gradient elution permits the sharp cutting of fractions, which emerge in an easily-removed volatile buffer that avoids extremes of pH values; CRAIG *et al.*[5] have pointed out the desirability of buffering the peptides for stability.

The procedure described in this paper has proved to be simple, reproducible, and adaptable to relatively large quantities. It requires a minimum of equipment; even the Autograd and the ultraviolet monitor are dispensable conveniences.

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SUMMARY

The A and B chains of performic acid-oxidized bovine insulin were separated by a rapid, simple procedure involving chromatography on DEAE-Sephadex. Elution was accomplished with a gradient of ammonium formate, which was removed from the final products by sublimation to yield salt-free preparations of the two peptides. The elution gradient produced good resolution of the A and B chains, which were collected in sharp fractions. The B chain emerged in a high state of purity, as judged by quantitative amino acid analysis. The A chain frequently contained traces of contamination which could be removed by rechromatography on the DEAE-Sephadex.

REFERENCES

1 S. M. MARTIN, K. SINGH, H. ANKEL AND A. H. KAHN, *Can. J. Biochem. Physiol.*, 40 (1962) 327.
2 G. PFLEIDERER AND G. SUMYK, *Biochim. Biophys. Acta*, 51 (1961) 482.
3 M. SATAKE, T. OMORI, S. IWANAGA AND T. SUZUKI, *J. Biochem.*, 54 (1963) 8.
4 T. MURACHI AND H. NEURATH, *J. Biol. Chem.*, 235 (1960) 99.
5 L. C. CRAIG, W. KONINGSBERG AND T. P. KING, *Biochem. Prep.*, 8 (1961) 70.
6 C. H. W. HIRS, S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 219 (1956) 623.
7 S. MOORE AND W. H. STEIN, *Methods Enzymol.*, 6 (1963) 819.
8 A. M. CRESTFIELD, S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 238 (1963) 622.
9 S. MOORE, *J. Biol. Chem.*, 238 (1963) 235.
10 C. ROLLINS, L. JENSEN AND A. N. SCHWARTZ, *Anal. Chem.*, 34 (1962) 711.
11 F. SANGER, *Biochem. J.*, 44 (1949) 126.
12 H. HAYASHI, *Seikagaku*, 32 (1960) 411; *C.A.*, 60 (1964) 3244a.
13 W. GRASSMAN, R. STROBEL, K. HANNIG AND M. DEFFNER-PLOCKL, *Z. Physiol. Chem.*, 305 (1956) 21.
14 M. J. MYCEK, D. D. CLARKE, A. NEIDLE AND H. WAELSH, *Arch. Biochem. Biophys.*, 84 (1959) 528.
15 J. L. BAILEY AND R. D. COLE, *J. Biol. Chem.*, 234 (1959) 1733.
16 G. H. DIXON AND A. C. WARDLAW, *Nature*, 188 (1960) 721.
17 S. FITTKAU, *Naturwiss.*, 50 (1963) 522.