AQUEOUS HEXAFLUOROACETONE: A SOLVENT FOR GEL PERMEATION CHROMATOGRAPHY OF LARGE PEPTIDES DERIVED FROM CHYMOTRYPSINOGEN A

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A distressing problem in protein chemistry has been the aggregation and insolubility of peptide fragments arising from various manipulations of proteins. These properties, particularly as they affect the separation of large oligopeptides, have seriously limited the scope of protein sequence studies. In the fall of 1964, reports appeared which suggested that certain highly fluorinated alcohols, pinacols, and gem-diols mixed with water might be useful in overcoming this problem (Middleton, 1964; Longworth, 1964). We have studied several of these solvents; one, hexafluoroacetone hydrate, has been developed as a useful aqueous system for the fragmentation and resolution of large peptides. This report presents application to studies of chymotrypsinogen and chymotrypsin involving cyanogen bromide cleavage and gel permeation chromatography.

MATERIALS AND METHODS

Hexafluoroacetone (HFA) was obtained from the Freon Products Division of E.I. duPont de Nemours and Company, Inc., Wilmington, Delaware, in the form of the "1.6 hydrate" ($HFA \cdot 1.6H_2O$). From this material the trihydrate ($HFA \cdot 3H_2O$) was prepared by addition of 1.4 moles of water per mole of solvent. The trihydrate is reported to distill, as such, at 105.5° from an undiluted solution (Richardson, 1966). The pK_a of HFA in dilute aqueous solution is 6.58 (Middleton, 1964); a pH of 1.9 has been recorded for 50% aqueous HFA solutions used in the course of the experiments reported here. The composition of HFA-water mixtures has routinely been determined by measurements of density since this property was found to be a linear function of volume percent $HFA \cdot 3H_2O$; the density of the trihydrate has been found to be 1.59 g/cm^3. Descriptions of other physical and chemical properties are given in duPont Technical Report DP-1, Freon Division.

The ultraviolet spectra of $HFA \cdot 3H_2O$ showed certain variations from lot to lot. All commercial samples examined prior to the purification described

below showed an absorption peak with a maximum at 279 nm and an accompanying high end absorbancy beginning at about 240 nm. (See Figure 1.) The peak at 279 nm did not obey Beer's Law at high concentration, but, in fact decreased very rapidly on dilution with water. The trihydrate was fractionally distilled from an 85-90% (V/V) aqueous solution through a 28-cm Vigreau column. The results of such a distillation are illustrated in Figure 1. The main fraction (about 80% yield) was the trihydrate recovered as a 98% aqueous solution distilling at 105°. Some of the contamination appeared in the first, more dilute aqueous fraction; the remainder did not distill and was found in the pot.

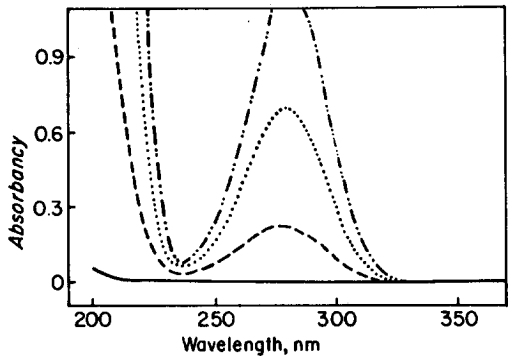

Figure 1. UV absorption spectra of HFA·3H_2O purified by distillation (———) and a commercial sample undiluted (— ·· —), diluted 1:1.3 (·····), and diluted 1:2 (— — —) with H_2O. Cary 15 Spectrophotometer, H_2O as reference, no N_2 purging.

HFA in solvents which have become contaminated in the course of laboratory experiments has been recovered in a pure state and in good yield by first distilling the solvent under vacuum in a rotary evaporator (Büchi apparatus with Teflon seals). The solvent was then directly distilled through a Vigreau column. Solvent with dissolved carbohydrate, protein, and other organic material has been handled in this manner.

Gel permeation chromatography was accomplished with Sephadex G-50 in Chromaflex chromatography columns (2.0 cm and 0.9 cm ID) which were purchased from Kontes Glass Company, Vineland, N. J. The perfluorinated solvents necessitated the use of solvent-resistant Vitron "O" rings and Chromaflex Teflon connecting tubing with KelF Luer unions, both of which are provided by Kontes. Absorbancy measurements of protein solutions and column effluents were carried out in quartz cells with a Beckman DU Spectrophotometer.

Cyanogen bromide (Eastman grade) was used directly as obtained from Eastman Organic, Rochester, N. Y. Ethylenimine, β-mercaptoethanol, guanidine hydrochloride, and 2-amino-2-methyl-1,3-propanediol were purchased from Matheson Cole, and Bell, Norwood, Ohio. Guanidine hydrochloride and the diol were multiply recrystallized from hot 95% ethanol and gave melting point ranges of 186-186.5° and 104-106°, respectively.

Chymotrypsinogen A (7x recrystallized; 0.04% chymotryptic impurity) was obtained from Princeton Laboratories, New Jersey, α-chymotrypsin (3x recrystallized) was purchased from Worthington Biochemical Corporation, Freehold, N.J.

The S-β-aminoethyl derivatives (AE-proteins) were prepared by a modification of the technique of Raftery and Cole (1966). Reaction took place at pH. 8.7 in 2 M 2-amino-2-methyl-1,3-propanediol buffer which was 5.6 M in quanidine hydrochloride. The protein was reduced with β-mercaptoethanol (final concentration, 0.15 M) and aminoethylated with a tenfold excess of ethylenimine. The solution was then extensively dialized against 0.1% β-mercaptoethanol, and the precipitated protein was recovered and lyophilized from 25% acetic acid.

RESULTS

Properties of Sephadex Gels in HFA. Microscopic examination of Sephadex gel beads equilibrated in 50% aqueous (V/V) HFA trihydrate showed swelling, extensive surface crazing, and a fragility greater than that in water. Columns which had been packed with care nevertheless gave relatively slow flow rates (e.g., 20 ml/hr for a 2.0 x 111 cm column with a hydrostatic head of 150 cm). No problem has been encountered either with deterioration of flow rate or resolution on Sephadex columns which have been in operation for several months. Bed volume and solvent regain (R_W and R_V) determined for G-50 using methods consistent with the suggestions and definitions of Flodin (1962) appear in Table 1.

TABLE 1

Solvent	Bed Volume	R_W	R_V
	ml/g	g/g	ml/g
H_2O	10.3	5.39	5.39
50%aq HFA·$3H_2O$	6.0	3.98	3.01

Cyanogen Bromide Cleavage of AE-Chymotrypsinogen and Resolution of Peptide Fragments. Reaction of the AE-zymogen with CNBr was conducted in 50% HFA·$3H_2O$ for 24 hours at room temperature. A 1% solution of the protein was adjusted to pH 1.0 by careful addition of concentrated HCl and reacted with a 75-fold excess of CNBr in a closed vessel. At the end of 24 hours the reaction solution was evaporated under vacuum at room

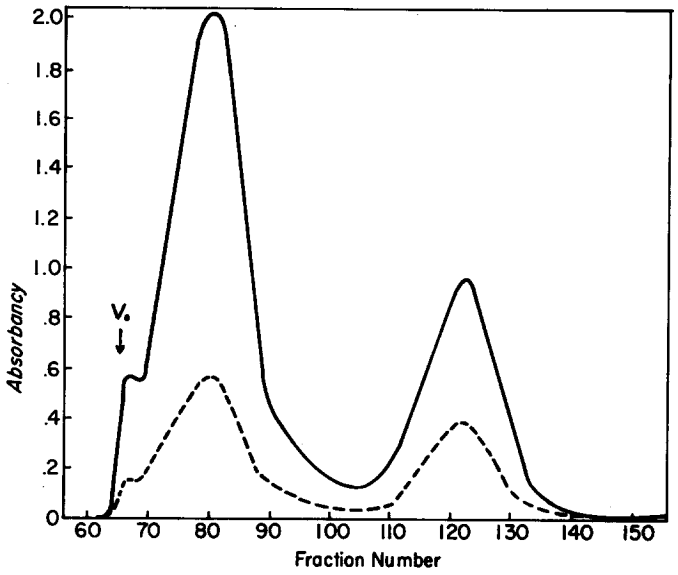

Figure 2. Gel permeation chromatography in 50% HFA·3H_2O of the CNBr peptides from AE-chymotrypsinogen. A_{230} (———) and A_{282}(— — —) vs. fraction number (2 ml volume). V_o = void volume.

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temperature. The horny, white residue quickly redissolved in 50% HFA·3H_2O and samples were applied directly to Sephadex columns.

Figure 2 shows the separation of 20.6 mg of product in a 2.0-ml sample passed through a G-50 column (2.0 x 11 cm) equilibrated in 50% HFA·3H_2O. Recovery was 98% based on absorbancy. Cleavage at one or both of the methionine residues of the protein would result in a large fragment, 180 or 192 residues in length, and a much smaller fragment consisting of the remainder of the chain (for sequence, see Hartley, 1964). These fragments correspond to the two main peaks of the chromatogram in the order and position of elution. The small leading peak is presumed to be the intact molecule. Using alanine content as an internal reference, amino acid analysis showed that the large fragment contained 1.8 residues of histidine and 5.5 residues of phenylalanine per 17 residues of alanine, whereas neither amino acid was found in the small fragment, in agreement with the known sequence. These values plus the levels of the other residues suggest that cleavage occurred in high yield at Met-192, but in poor yield at Met-180. Furthermore, no small peptide representing residues 181-192 was evident in the chromatogram determined by absorbance at 230 mm. The absence of methionine in the amino acid analysis of both fragments remains to be explained.

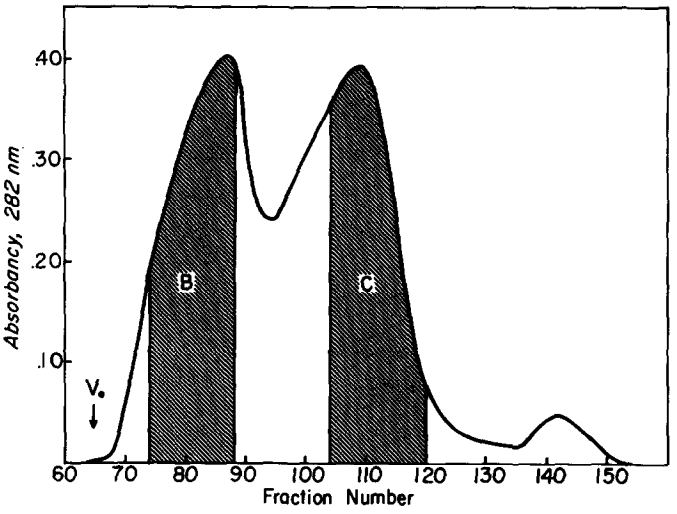

Figure 3. Gel permeation chromatography in 50% HFA·$3H_2O$ of the B- and C-chains of AE-α-chymotrypsin. The 2-ml fractions were pooled for amino acid analyses as indicated. V_o = void volume.

Resolution of the B- and C-chains of AE-α-Chymotrypsin. α-Chymotrypsin was aminoethylated in the fashion described above; lyophilization was accomplished from an opalescent solution (< 5 mg/ml) in 25% acetic acid. Thy lyophilized material dissolved instantly in 98% HFA·$3H_2O$ (35 mg/ml) was applied to the G-50 column (2.0 x 111 cm) equilibrated with the same solvent. The chromatogram is presented in Figure 3. The effluent volumes for the two peaks were those expected for the B-chain (residues 16-146 and the C-chain (residues 148-245). Furthermore, the ratio of total absorbances at 282 nm for the two peaks and the amino acid analyses were consistent with this identification (Hartley, 1964). Based on alanine content, 1.9 residues of histidine and 6.7 residues of phenylalanine per 11 alanines were found in the first peak, whereas no methionine was evident. Approximately one-half a residue of phenylalanine (suggesting 8% contamination with B-chain), both methionines, and no histidine were found in the second peak.

DISCUSSION

The ready solubility of large fragments of the chymotrypsinogen chain in the HFA-water system and their resolution on Sephadex columns according to size demonstrate the power of the new solvent. These results may be contrasted to earlier work with different solvent systems; in those studies a facile separation of the B- and C-chains was not possible because the strong tendency toward aggregation impeded the efficient resolution of the chains (Van Hoang, et al., 1963; Hartley, 1964; Richmond, 1966).

HFA-water systems have other useful properties. Peptides can be monitored down to 210 nm since the pure solvent is optically transparent, and peptides can be readily recovered from chromatographic fractions by evaporation of the solvent under vacuum. The solvent action probably depends on the weak acidity and strong hydrogen bond forming properties of the HFA hydrate. The solvent is known to be a strong denaturing agent for the α-helix conformation (Longworth, 1964). It should be useful as a medium for specific chemical cleavage, as shown in the present case of the action of cyanogen bromide on chymotrypsinogen.

Acknowledgements

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