Journal of Chromatography, 266 (1983) 511–522
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 034

TWO-DIMENSIONAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND CHEMICAL MODIFICATION IN THE STRATEGY OF SEQUENCE ANALYSIS

COMPLETE AMINO ACID SEQUENCE OF THE LAMBDA LIGHT CHAIN OF HUMAN IMMUNOGLOBULIN D

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SUMMARY

Rapid high-performance liquid chromatographic procedure forms part of the strategy for determining the amino acid sequence of proteins. It was applied to the light chain of an immunoglobulin D, and the result, together with previous data on the sequence of the heavy chain, established the complete covalent structure of a human IgD immunoglobulin. We aimed for rapid purification of both small and large peptides. The two-step chromatography system ("two-dimensional chromatography") consists of a combination of macroreticular cation-exchange chromatography as the first dimension and reversed-phase chromatography as the second. This procedure was used for the systematic separation of the tryptic peptides of the light chain. In order to obtain overlapping of the tryptic peptides, chemical modifications of the light chain and reversed-phase chromatography were found to be the most reliable path toward the sequence analysis and greatly accelerated the determination of the light chain.

INTRODUCTION

The increased demand for amino acid sequence data as a basis for the understanding of protein structure and function and genetic control has spurred the development of more rapid and sensitive techniques. Edman degradation has become the chief method for determining amino acid sequences, and specific cleavage of the protein and isolation of the resulting peptides are the rate-determining steps in the determination of the complete primary structure of a protein. New chemical reagents and enzymes for several methods[1,2] of specific protein cleavage are now commercially available, but the greatest advance has been made in the field of peptide separation by reversed-phase high-performance liquid chromatography (RP-HPLC). This method has allowed complete separation of peptide mixtures, such as the tryptic digest of a small protein, *e.g.* bovine intestinal calcium-binding protein[3]. It has also permitted the resolution of less complex mixtures of large peptides, such as the cy-

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anogen bromide fragments of hemoglobin[4]. However, when it is applied to separation of very complex mixtures of peptides, RP-HPLC alone is normally not sufficient to resolve all of them. Often, to obtain overlapping peptides, cleavage methods that are not highly specific or that give low yields must be selected in the strategy of sequence analysis even though the resulting peptide mixture may be very complex. In such cases conventional separation techniques such as Sephadex gel chromatography have been used as a preliminary to RP-HPLC purification in order to simplify complex peptide mixtures[5]. However, such methods not only require much more material than does HPLC, but also give insufficient resolution.

The use of two-step chromatographic systems has been described in the literature, including various combinations of techniques. For example, Kratzin *et al.*[6] applied reversed-phase chromatography to the separation of light-chain peptides by using first one buffer system and then another, but they used a single type of column under changed conditions. Fürst *et al.*[7] adapted an automatic valve-change system to a two-step chromatography procedure. Axelson *et al.*[8] developed a procedure for profile analyses of aromatic compounds by a combination of ion-exchange chromatography and gas chromatography–mass spectrometry.

In this paper we describe the purification of tryptic peptides of the light chain from human immunoglobulin D (IgD) by a technique we call high-resolution two-dimensional chromatography. This technique involves a combination of macroreticular cation-exchange column[9] chromatography and reversed-phase chromatography, followed by extensive application of HPLC to the purification of overlapping peptides obtained by chemical modifications of the protein.

EXPERIMENTAL

Materials

The lambda light chain of IgD protein WAH was purified as described by Lin and Putnam[10]. It was pure as judged by several criteria, including polyacrylamide gel electrophoresis with sodium dodecyl sulfate. L-(Tosylamido-2-phenyl)ethylchloromethyl ketone (TPCK)-treated trypsin (E.C. 3.4.21.4) was purchased from Worthington (Freehold, NJ, U.S.A.). Pyroglutamate aminopeptidase (E.C. 3.4.11.8) was obtained from Boehringer Mannheim (Castleway, IN, U.S.A.).

The chemicals used were methanesulfonic acid (Aldrich, Milwaukee, WI, U.S.A.), ammonium hydroxide (Ultrapure) (J. T. Baker, Phillipsburg, NJ, U.S.A.), acetonitrile and 1-propanol (chromatography grade) (Burdick & Jackson, Muskegon, MI, U.S.A.), and trifluoroacetic acid (TFA) (Pierce, Rockford, IL, U.S.A.).

High-performance liquid chromatography

The macroreticular cation-exchange resin, Hitachi gel 3013C (5–7 μm), was kindly provided by Dr. S. Ganno of Hitachi Ltd. (Tokyo, Japan). A reversed-phase Synchropak RP-P column (25 × 0.41 cm I.D.) was obtained from Synchrom (Linden, IN, U.S.A.).

The Hitachi resin was packed into a jacketed stainless-steel column (25 × 0.41 cm I.D.) as described by Isobe *et al.*[9]. The solvent was delivered by a Beckman pump, Model 110A, and the eluate was monitored at 210 nm in a 1-cm light path with a Gilson Holochrome UV monitor, connected to a Linear recorder, Model 261/MM. The programmed gradient was formed by a Beckman controller Model 421 system.

Chemical modifications and tryptic digestion

The aminoethylated light chain[10] and the light chain modified as described below (2.5–5 mg) were digested with TPCK-treated trypsin (50–100 μg) in 0.1 *M* ammonium bicarbonate at 37°C for 6 h. The modification of lysine residues of the light chain with citraconic anhydride was carried out by the method of Atassi and Habeeb[11]. After tryptic digestion of the citraconylated light chain, the digest was treated with 20% formic acid overnight to deblock the modified lysine residues. The aminoethylated light chain was also modified at arginine residues with cyclohexane-1,2-dione by the method of Patthy and Smith[12]. Tryptic digestion of this was followed by treatment with 0.5 *M* hydroxylamine to remove dihydroxycyclohexylene from the modified arginine.

Purification of tryptic peptides by two-dimensional high-performance liquid chromatography

The lyophilyzed tryptic digest (1 mg in 100 μl of 0.4% ammonium hydroxide) was applied to a cation-exchange column of Hitachi gel 3013C and eluted at a flow-rate of 0.5 ml/min with a gradient programmed from distilled water to the buffer designated B [0.4 *M* ammonia, 0.005 *M* acetic acid containing 50% (v/v) acetonitrile and 25% (v/v) 1-propanol, adjusted to pH 6.2 with methanesulfonic acid]. The column temperature was maintained at 70°C. After each analysis, the column was washed with B, containing 0.1 *M* acetic acid until the pH of the eluate became 6.2; the column was then re-equilibrated with water for 30 min before the next sample was introduced. The above conditions were modified from those of Isobe *et al.*[9]. The eluate fractions from the Hitachi column were pooled appropriately and lyophilyzed. Aliquots, dissolved in 0.1% TFA or 4 *M* guanidine hydrochloride, were applied to the reversed-phase column and eluted for 40 min at a flow-rate of 0.7 ml/min with a linear gradient from 0.1% TFA to 40% 1-propanol, containing 0.1% TFA.

Purification of lysine- or arginine-overlapping peptides

The tryptic digest of the citraconylated light chain was deblocked with 20% formic acid and fractionated by gel chromatography on a Sephadex G-50 column (60 × 1.5 cm I.D.) pre-equilibrated with 0.1% TFA containing 10% 1-propanol. The peptides were purified further by HPLC on the reversed-phase column with a linear gradient from 0.1% TFA to 60% 1-propanol, containing 0.1% TFA, at a flow-rate of 0.7 ml/min for 75 min.

The tryptic digest of the light chain modified with cyclohexanedione was treated with 0.5 *M* hydroxylamine, then desalted by gel filtration on a Sephadex G-25 column (60 × 1.5 cm I.D.) and applied to the reversed-phase column.

Amino acid and sequence analyses

The methods for amino acid analysis with the Beckman amino-acid analyzer (Model 121M) and for sequence determination with the Beckman sequencer (Model 890C) have been described[13,14]. The amino-terminal peptide was blocked, therefore it was treated with pyroglutamate peptidase[15].

Nomenclature of peptides

The nomenclature of peptides of the WAH lambda light chain follows that

adopted for other human lambda chains[16,17]. Tryptic peptides are given the prefix T and are identified by the letter V for the variable region and by λ for the constant region of the sequence. Since the protein was aminoethylated, additional tryptic peptides ending with aminoethylcysteine were obtained; these are given the same number as the tryptic peptide expected from the unsubstituted protein plus the letter "a", and the rest of the tryptic peptide has the same number plus the "b". The peptides in the variable region of the protein are numbered consecutively in their order in the sequence. The peptides in the constant region are numbered consecutively beginning with Tλ1 (which is hypothetical). The tryptic peptides from the protein modified with citraconic anhydride or cyclohexanedione are also given the prefix T and are identified by the letters "ci" for the peptides from the citraconylated protein and "cy" for the peptides from the light chain modified with cyclohexanedione. These peptides are numbered consecutively in their order in the sequence. (For a summary of the nomenclature and the ordering of the peptides see Fig. 7.)

RESULTS AND DISCUSSION

Purification of the tryptic peptides of the lambda light chain of human IgD by two-dimensional chromatography

The tryptic digest of the aminoethylated light chain was first fractionated by macroreticular cation-exchange column chromatography (Fig. 1), and each of the twenty fractions eluted from the column was then applied automatically to the reversed-phase column. The profile of all of the chromatograms is shown in Fig. 2 as a three-dimensional visualization. This visualization facilitates the summarizing of the tryptic peptide analysis and aids recognition of overlapping peaks. All the tryptic

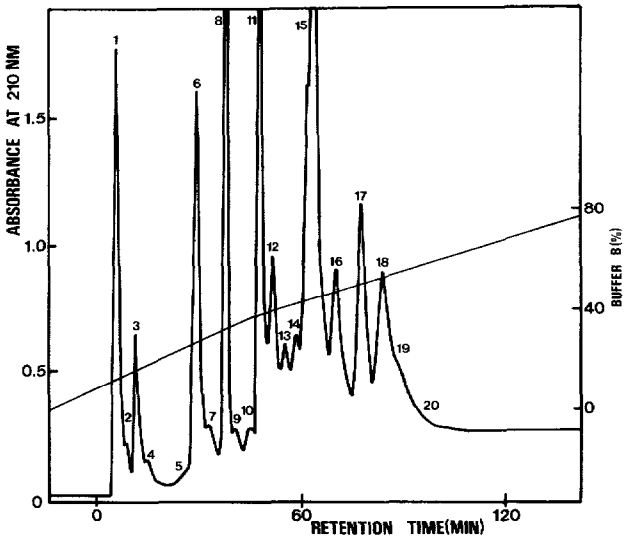

Fig. 1. First fractionation of the peptides of the tryptic digest (750 μg) of the aminoethylated lambda light chain of human IgD WAH on a macroreticular cation-exchange Hitachi gel 3013C column. Fractions in each peak were pooled, and the material was applied to a reversed-phase column (see peaks 1–20 in Fig. 2).

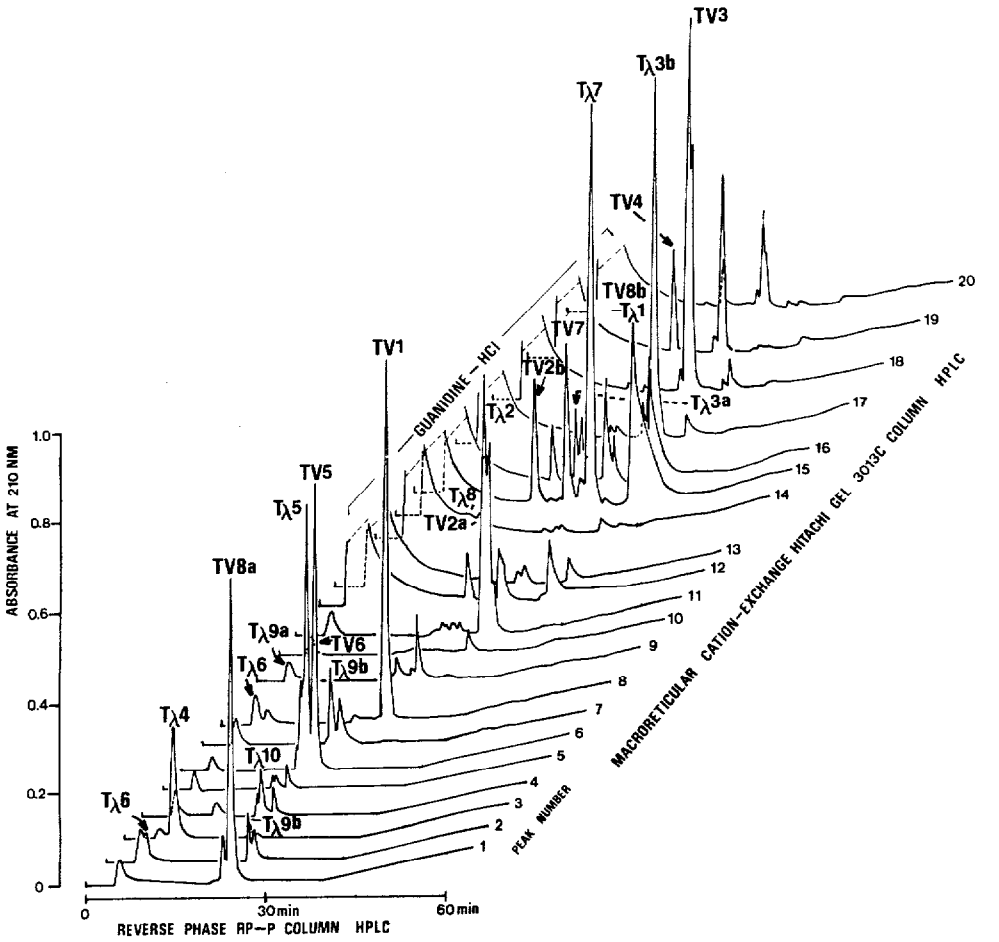

Fig. 2. A three-dimensional visualization of the chromatograms of peaks 1–20 of Fig. 1 on a reversed-phase Synchropak RP-P column as the second dimension. The nomenclature for peptides described in Experimental is used to indicate the elution positions of the tryptic peptides.

peptides from the protein were purified by this two-step system we call "high-resolution two-dimensional chromatography", and each peptide is identified in Fig. 2 by its elution position. The yields, net charges, and hydrophobicities of the peptides are listed in Table I in the order of elution from the macroreticular cation-exchange column.

Although the elution conditions were slightly different from those of Isobe *et al.*[9], the order of elution of the peptides of the constant region of the WAH lambda light chain from the macroreticular cation-exchange column almost corresponded to that of a different lambda light chain described by Isobe *et al.* However, the elution orders of the peptides of the variable region of the two light chains were very different. The net charges of the tryptic peptides expected at pH 6.2, where the peptide amino groups should be charged, ranged from -3 to $+2$ (Table I). The values of hydrophobicities of the peptides, which were calculated by using Rekker's hydropho-

TABLE I

SUMMARY OF THE NET CHARGES, HYDROPHOBICITIES, AND YIELDS OF THE TRYPTIC PEPTIDES

The peptides are shown in the order of elution from the macroreticular cation-exchange column (Fig. 1). Peptides Tλ9b and Tλ6, which had a glutamine residue as N-terminal, were obtained in two forms, one of which was probably cyclyzed during the chromatography.

	Peak number										
	1	*2*		*3*	*4*	*6*		*7*	*8*		*9*
Peptides	TV8a	Tλ9b	Tλ6	Tλ4	Tλ10	Tλ5	TV5	Tλ9b	TV1	Tλ6	Tλ9a
Total residues	9	11	5	7	8	10	11	11	17	5	4
Charge	−3	−1	0	0	0	0	0	0	0	+1	+1
Hydrophobicity	3.71	1.92	−2.21	2.38	2.37	2.11	2.14	0.90	3.26	−3.23	1.10
Yield (%)	100	20	52	100	49	93	83	55	100	30	62

bic fragmental constant[18], ranged from −3.23 to +15.3 (Table I). Although strong interactions between peptides and a macroreticular resin of the styrene–divinylbenzene type are expected to occur during chromatography, all the peptides were recovered at yields of from 41% to 100% (Table I). The peptide eluted in lowest yield was TV3; this peptide has the third highest value of hydrophobicity (12.4) and has the aromatic N-terminal sequence of Tyr-Tyr-Val-Tyr-Trp-Tyr. Other highly hydrophobic peptides, TV8b-Tλ1 (15.3), Tλ3b (14.9) and Tλ7 (9.18), were recovered in yields of 79%, 65% and 76%, respectively, although the peptides TV8b-Tλ1 and TV3 could not be obtained by paper chromatography (unpublished data).

The mechanism of separation by macroreticular cation-exchange chromatography seems to involve not only electrostatic interaction, but also hydrophobic interaction between peptides and the polystyrene resin. For, even if the values of hydrophobicity of the peptides are high and close together, they are still eluted almost in the increasing order of their net positive charge (Table I). However, peptides with high hydrophobicity tend to be eluted later, though not exactly in the order of increasing hydrophobicity. On the other hand, the mechanism of separation by reversed-phase chromatography involves mainly the hydrophobic interaction between the column packing and peptides. O'Hare and Nice[19] observed that the retention time of peptides smaller than fifteen residues is linearly related to the sum of Rekker's constant for constituent amino acids in a linear gradient elution system. This relationship was also observed in our system of separation with reversed-phase column chromatography as the second dimension. The small deviation from the postulated relationship may be explained by the suggestion of Okuyama and Sasagawa[20] that larger peptides have some secondary structure instead of just the random structure characteristic of small peptides.

The resolving power of the macroreticular column is as good as that of the reversed-phase column[9,21]. The combination of two types of HPLC, each with high resolving power but based on a different separation mechanism, provides much better resolution of very complex peptide mixtures than does either method alone. The

10	*11*	*14*		*15*				*16*	*17*	*18*	*19*
TV6	Tλ2	TV2a	Tλ8	TV2b	TV7	Tλ7	TV8b-Tλ1	Tλ3a	Tλ3b	TV3	TV4
5	19	5	3	9	13	15	23	5	15	15	5
+1	−1	+1	+2	+1	+1	0	−1	+1	0	+1	0
1.64	8.60	3.15	−0.27	2.20	5.26	9.18	15.3	4.24	14.9	12.4	8.19
70	80	67	70	65	80	76	79	70	65	41	55

advantages of the macroreticular column, which include a relatively high sample capacity and also stability under alkaline conditions[9,21] make it preferable for the initial separation of a complex peptide mixture such as an enzymatic digest; its high sample capacity is essential for obtaining enough pure peptide for sequence analysis, and its high stability permits washing with sodium hydroxide even after much use, which is important for reproducibility. The use of reversed-phase chromatography as the second purification method also has several advantages. One is that the column efficiency is increased by the reduction in the number of peptides in the first separation step. Reversed-phase chromatography also removes the salt from the first step, and this allows use of a buffer system with low absorbance at 210 nm. Furthermore, even if the peptides from the first separation become insoluble, a strong denaturing solvent, such as guanidine–HCl, can be used to dissolve them. In fact, 4 *M* guanidine–HCl was used to dissolve the peptides from peaks 12–20 in Fig. 1.

The purified tryptic peptides were sequenced by automated Edman degradation as described elsewhere[22]. Because the amino-terminal peptide TV1 was blocked, it was treated with pyroglutamate peptidase and the products were separated by HPLC (Fig. 3). This approach allowed the identification of amino-terminal pyroglutamate as glutamic acid and also the determination of the sequence of the peptide. The yields of glutamic acid and the remaining peptide were *ca.* 90%.

Purification of lysine- and arginine-overlapping peptides from chemical modifications of the light chain

The purification of the tryptic peptides of the aminoethylated light chain was highly systematized by the two-dimensional chromatography. The method may also be useful for peptide mapping of large proteins. Although the amino acid sequences of all the tryptic peptides were determined, the peptides from a single enzymatic digest are not sufficient to complete the sequence of any protein, because of the need for overlapping the peptides. However, the tryptic peptide sequences provided the information needed to select the best cleavage method for complete sequencing of

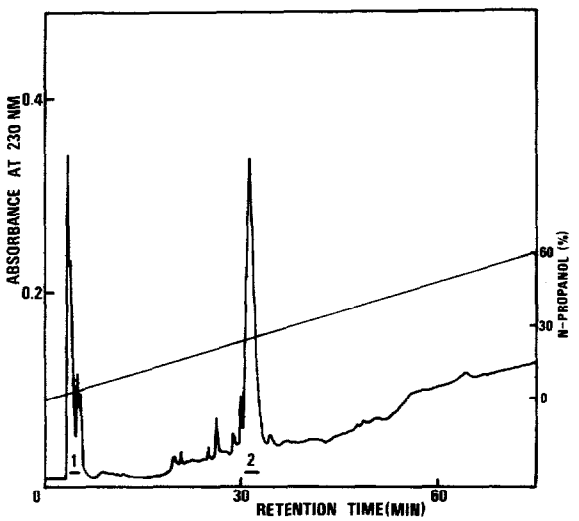

Fig. 3. Separation on the reversed-phase column of the pyroglutamate peptidase digest of the blocked N-terminal peptide (TV-1). The pyroglutamic acid removed from the peptide was eluted at the position of fraction 1. Fraction 2 contained the deblocked N-terminal peptide.

the protein. The lack of methionine precluded cleavage with CNBr, and the absence of Asn–Gly and Asp–Pro peptide bonds prevented the use of hydroxylamine or acid cleavage, respectively. The strategy chosen to obtain overlapping peptides was based on our ability to purify large peptides by use of HPLC. Thus, we based our strategy on two results established by our sequence analysis of the tryptic peptides: (i) although there are five arginines in this lambda light chain, only four tryptic peptides containing arginine were found because one contained an Arg–Pro bond; (ii) this light chain proved to be the Oz^+ isotype in which Arg is replaced by Lys at position

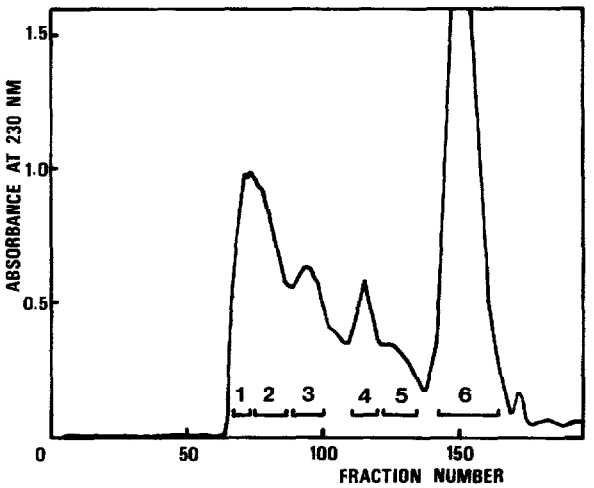

Fig. 4. Separation of the tryptic digest of the citraconylated light chain by gel filtration on a Sephadex G-50 column. Peptides were pooled as indicated by horizontal bars.

191[22] (Oz isotype is a polymorphism in the constant region of the lambda light chain). Hence, we predicted that four smaller peptides and one large peptide containing the entire constant region should be obtained on tryptic digestion of the citraconylated protein.

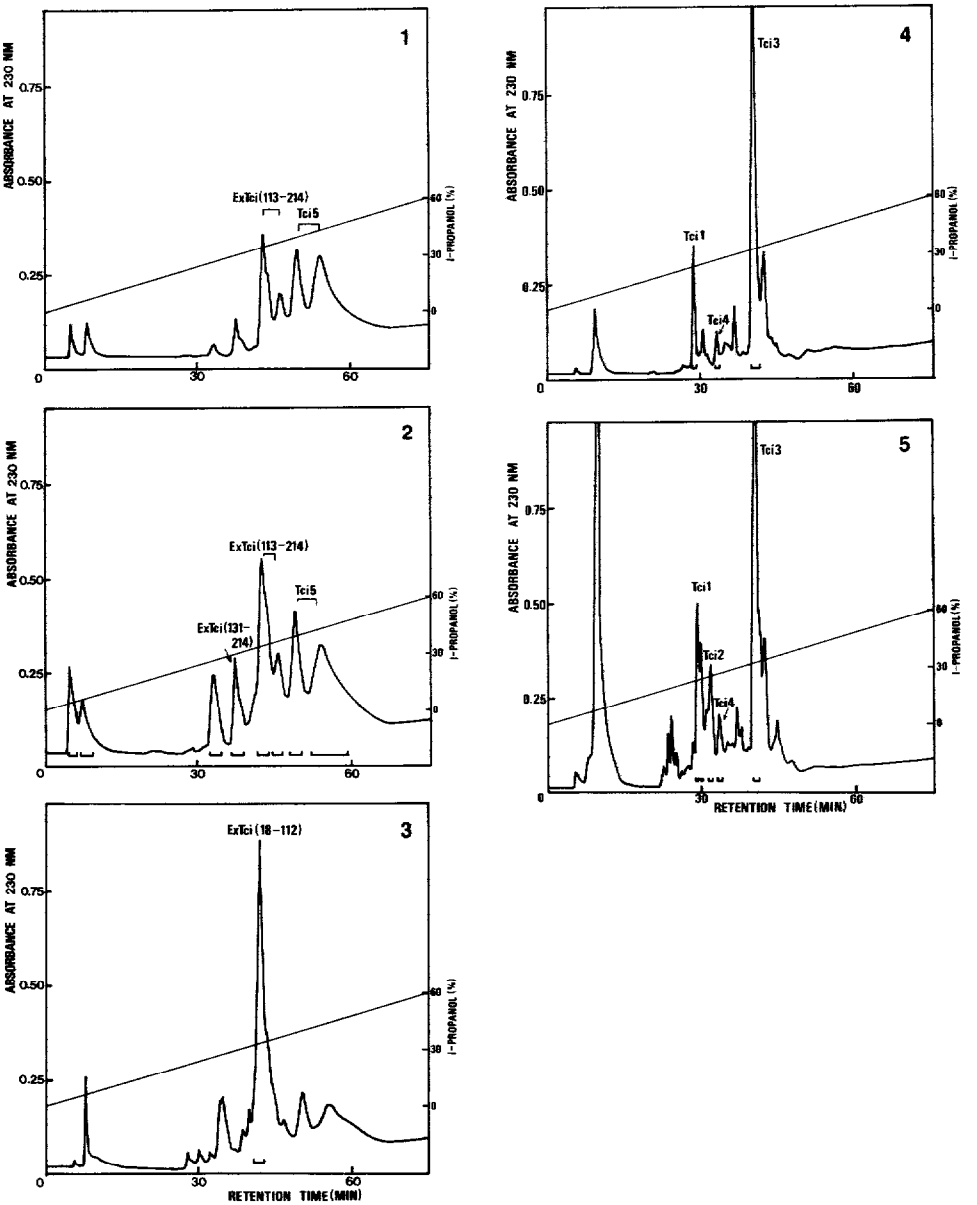

Fig. 5. Chromatograms of the peptide pools 1–5 from Fig. 4 obtained by reversed-phase chromatography on a Synchropak RP-P column. The number at the top right of each chromatogram gives the fraction number from Fig. 4. The elution positions of the expected citraconylated tryptic peptides are indicated by Tci and those from unexpected cleavage by ExTci. Numbers in parentheses give the position of the peptide in the protein sequence.

The tryptic digest of the citraconylated light chain was first fractionated by gel chromatography (Fig. 4) because of the wide range of molecular weights expected. Then, each fraction in Fig. 3 was purified by RP-HPLC as shown in Fig. 5. Five main peptides, produced by the cleavage at the expected sites, were obtained, and also many minor components (Fig. 5). Four of the main peptides (Tci1, Tci2, Tci3, and Tci4) were recovered in high yield (97%, 55%, 72%, and 61%, respectively), but the yield of peptide Tci5 was low (23%). However, peptides ExTci(113–214), ExTci(131–214) and ExTci(18–112), which were derived from the region within Tci5 or from the region containing a part of Tci5, were also obtained in yields of 11%, 4% and 11%, respectively (Fig. 5). Because peptide Tci5 contained the entire constant region and had nine lysines, any incomplete modification would cause considerable heterogeneity in the peptides produced. Furthermore, the overlapping sequences and size of the incompletely modified peptides would greatly complicate their separation, especially for large peptides. Nonetheless, Tci5, which began in the variable region and included the entire constant region (residues 81–214) was purified by the use of RP-HPLC, as were the other lysine-overlapping peptides. The five main Tci peptides were eluted approximately in the order of increasing hydrophobicity and had separate elution positions in the chromatogram. However, many extra peptides were generated because of incomplete modification. Therefore, a one-step purification of the peptides was not possible and gel filtration had to be used for the first separation procedure. The results of the sequence analysis of the lysine-overlapping peptides are described in a separate paper[22].

In the next stage, the arginine residues were blocked with cyclohexanedione in order to obtain arginine-overlapping peptides. After tryptic digestion of the modified light chain, the digest was treated with hydroxylamine to remove dihydroxycyclo-

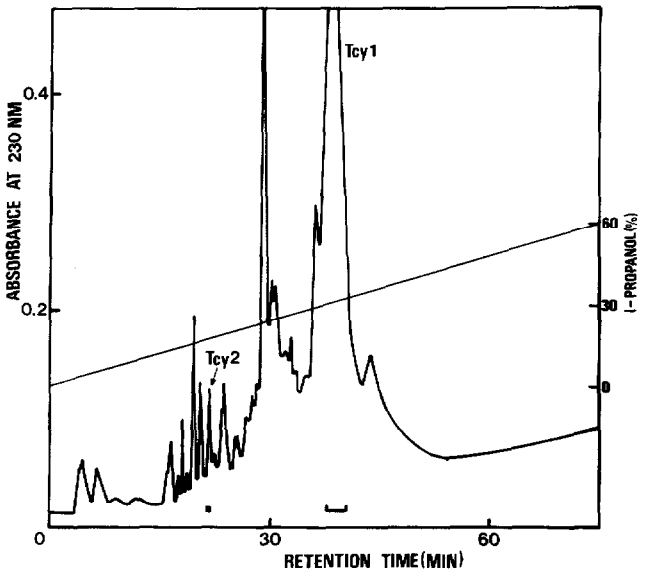

Fig. 6. Purification on the reversed-phase column of the arginine-overlapping peptides from the light chain modified with cyclohexanedione. The elution positions of the arginine-overlapping peptides are indicated by Tcy1 and Tcy2. The peptides were pooled as indicated by horizontal bars.

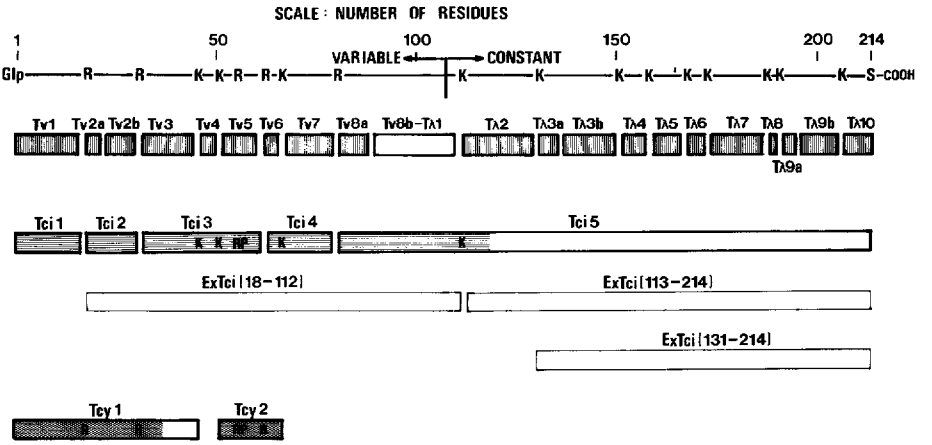

Fig. 7. Summary of the results of sequence analysis of the lambda light chain of human IgD WAH. The symbols for peptides described in Experimental are used in this figure, and the one-letter code is used for amino acid residues. The shading indicates the portions of the peptides for which the amino acid sequence was determined[22].

hexylene from the modified arginine residues[12]. Because all of the arginine-overlapping peptides were expected to be larger than sixteen residues, Sephadex G-25 gel filtration was selected for preliminary separation and for the removal of hydroxylamine. Only the Sephadex fraction expected to contain peptides with more than sixteen residues was applied to a reversed-phase column (Fig. 6). Because tryptic cleavage at the aminoethylcysteine residue (Cys-22) did not occur, the N-terminal peptide, Tcy1, which had 46 amino acid residues including two arginine residues was obtained, and this peptide gave two arginine-overlapping sequences[22]. Peptide Tcy2 with sixteen amino acid residues also had two arginines. One of these was in an Arg–Pro bond resistant to trypsin; the second arginine gave another overlapping sequence[22]. These two peptides accounted for four of the five arginines. Although the peptide containing the fifth arginine (Arg-80) was not isolated, the results described gave enough evidence to construct the entire protein sequence. (However, to obtain independent proof of the order of the peptides, we obtained the complete overlapping sequence data including the constant region by sequence analysis of *Staphylococcus aureus* protease peptides of the light chain, which were also purified by HPLC[22]. The ordering of the peptides in the final sequence of the protein, based in results described here, is given in Fig. 7.

CONCLUSIONS

The strategy for the sequence analysis was designed to take the shortest possible course by extensive use of HPLC for the purification of both small and large peptides. In the first step, the tryptic peptides of the light chain were purified systematically by two-dimensional chromatography. In the second stage, chemical modifications of the protein were selected to produce peptides that would overlap the tryptic peptides. The choice of methods was based on their reliability and on the results of sequence analysis of the tryptic peptides. Tryptic digestion of the modified

protein simplified the separation scheme and the ordering of the peptides to give the final protein sequence. The flexibility, speed and high resolving power of HPLC allow its application at almost every stage in the strategy of protein sequence analysis. The sequence determination of this light chain was completed in a few months, which compares favorably with the rate of nucleotide sequence analysis of the DNA encoding a light chain. By combining the results of the sequence determination of this light chain with our previous report of the complete sequence of the delta heavy chain[14], we were able to establish the complete covalent structure of a human IgD immunoglobulin.

ACKNOWLEDGEMENTS

We thank Dr. S. Ganno of Hitachi Ltd. for donation of Hitachi gel 3013C and Dr. T. Okuyama for valuable suggestions. We also thank S. A. Dorwin, P. H. Davidson, J. Madison, and K. Huss for their excellent technical assistance. This work was supported by grants IM-2H from the American Cancer Society and CA08497 from the National Cancer Institute.

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