Journal of Chromatography, 326 (1985) 407–418
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 535

AUTOMATED TANDEM HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM FOR SEPARATION OF EXTREMELY COMPLEX PEPTIDE MIXTURES

NOBUHIRO TAKAHASHI*, NORIAKI ISHIOKA, YOKO TAKAHASHI and FRANK W. PUTNAM
Department of Biology, Indiana University, Bloomington, IN 47405 (U.S.A.)

SUMMARY

We have developed an automated tandem chromatography system, which consists of a combination of ion-exchange column chromatography and reversed-phase column chromatography. The system is composed of two independent high-performance liquid chromatography assemblies, in each of which programmed elution is carried out by a computer-assisted controller. A peptide mixture is applied to an ion-exchange column and is eluted in a stepwise manner. The eluent from the first column is introduced directly into the second, reversed-phase column, which is connected in tandem through a tee tube. After application of two column volumes of the eluent, reversed-phase chromatography is performed by linear gradient elution. Stepwise elution for ion-exchange chromatography and the gradient elution for reversed-phase chromatography are synchronized by a computer program. The resolving power and the reproducibility of the method were tested by using a tryptic digest of human ceruloplasmin [molecular weight 132 000 daltons (132 kDa)]. By this method, the digest was resolved reproducibly into several hundred peaks within 16 h. All of the four glycopeptides expected to be obtained by tryptic digestion were purified easily from the whole digest of the protein. Comparison of the peptide maps between a single-chain and a degraded form of ceruloplasmin facilitated the identification of two tryptic peptides, derived from the carboxyl-terminal regions of 67 kDa and 50 kDa fragments of the degraded form, which lack the carboxyl-terminal arginine and lysine residues, respectively. The method may be applicable to comparative peptide mapping of very large proteins exhibiting molecular microheterogeneity, such as carbohydrate or genetic variants; it also can be used complementarily for sequence support of DNA sequencing as well as for preparative purification of peptides as a strategy of protein sequencing of very large proteins.

INTRODUCTION

Human plasma proteins, of which *ca.* 100 have been identified, mainly have high molecular weights (above 50 kilodaltons, kDa), and often exhibit microheterogeneity[1]. The latter may occur during post-translational processing of the gene-

0021-9673/85/$03.30

derived polypeptide, such as incomplete glycosylation, proteolytic cleavage etc.[2], or it may be due to genetic variation or polymorphism in some populations or in some diseases[1]. Although the complete amino acid sequences of *ca.* 50 plasma proteins have been determined[1], the molecular basis of the heterogeneity is not yet known in many proteins because of the large size of the molecules.

Recent progress in high-performance liquid chromatography (HPLC) has greatly improved the efficiencies of the peptide separation and of other analytical methods in the field of protein chemistry[3,4]. However, efforts are still being made to improve the resolving power and to decrease the amount of sample needed for analysis. Peptide maps are used, both for comparison of closely related proteins and for isolation of peptides generated by various enzymatic or chemical cleavages. Reversed-phase HPLC (RP-HPLC) has been used for peptide mapping to identify amino acid substitution or carbohydrate variants in some molecules[5,6]. However, RP-HPLC alone is normally not sufficient to resolve a complex peptide mixture from a large protein. Tandem HPLC peptide mapping gives much higher resolution and is very useful for separating complex peptide mixtures. Gel filtration, followed by RP-HPLC[7], a cation-exchange column with RP-HPLC[8], RP-HPLC at two different pH values[9], and RP-HPLC with two different ion-pairing agents[10] have all been used for tandem HPLC mapping. These procedures are usually carried out manually; *i.e.*, the eluent from the first column is collected in many fractions and then each fraction is applied to the second column separately. However, manual operation of tandem HPLC may cause many problems such as precipitation during concentration of peptide fractions, sample loss by multistep handling, increased time and labor, chance of human error, and decreased reproducibility through accumulation of experimental errors.

In this report, we describe an automated tandem chromatography system for peptide mapping of very large proteins. For automation of tandem HPLC, it is essential to introduce the eluent from the first column into the second column without pooling eluent fractions and/or pretreatment. The automated tandem chromatography system consists of a combination of anion-exchange column chromatography and reversed-phase column chromatography. The system is composed of two independent HPLC assemblies, in each of which programmed elution is performed by a computer-assisted controller. In this system, a peptide mixture is applied first to an anion-exchange column and is eluted in a stepwise manner. The eluent from the first column is introduced directly into a second, reversed-phase column, which is connected in tandem through a tee tube. After application of the eluent, reversed-phase chromatography is performed by a linear gradient elution. Stepwise elution for anion-exchange chromatography and gradient elution for reversed-phase chromatography are synchronized by computer programs. By this method, a tryptic digest of human ceruloplasmin with M_r = 132 000 was resolved reproducibly into several hundred peaks within 16 h.

EXPERIMENTAL

Materials

Two preparations of human ceruloplasmin, purified from normal pooled plasma, were used. One preparation was undegraded single-chain ceruloplasmin with a

molecular weight of 132 000 (132 kDa) and was prepared by the method of Noyer *et al.*[11]. The second had undergone autolytic proteolysis and was a mixture of three main fragments with approximate molecular weights of 19, 50, and 67 kDa[12]. These preparations, whether single-chain or spontaneously cleaved, consisted predominantly of Type I ceruloplasmin with a small amount of Type II. The only difference between Type I and Type II is believed to be that Type I is glycosylated at position Asn-339, whereas Type II is not[7,13]. Also used were 3.1S leucine-rich α_2-glycoprotein and α_1B-glycoprotein, obtained from Behringwerke (Marburg/Lahn, F.R.G.).

The sources of enzymes, reagents and columns were: trypsin, treated with L-(tosylamido-2-phenyl)ethylchloromethyl ketone (TPCK), chymotrypsin (Worthington, Freehold, NJ, U.S.A.), trizma base (Sigma, St. Louis, MO, U.S.A.), methanesulfonic acid (Aldrich, Milwaukee, WI, U.S.A.), ammonium hydroxide (Ultrapure) (J. T. Baker, Phillipsburg, NJ, U.S.A.), trifluoroacetic acid (TFA) (Pierce, Rockford, IL, U.S.A.), acetonitrile (chromatography grade) (Burdick & Jackson, Muskegon, MI, U.S.A.). A Spherogel-TSK IEX-540 DEAE column (30 × 0.4 cm I.D.) and an Ultrasphere ODS column (15 × 0.4 cm I.D.) were both from Altex, Berkeley, CA, U.S.A.

Tryptic digestion

The carboxymethylated proteins (5–10 mg) were digested with TPCK-treated trypsin (50–100 μg) in 0.1 *M* ammonium bicarbonate at 37°C for 12 h. The digest was lyophilized to remove the ammonium bicarbonate, and the lyophilizate was dissolved in 1 ml of 0.02 *M* Tris-acetic acid buffer (pH 8.0). The insoluble material was removed by centrifugation, and the supernatant was used as a sample for tandem chromatography.

Apparatus

The HPLC apparatus used for the first-column chromatography was a Model 334 Gradient HPLC System (Beckman, Berkeley, CA, U.S.A.) including a 421 CRT controller, two 110A pumps, a 210A injector, and a mixing chamber. A Model 344 Gradient HPLC System, including a 421 CRT controller, two 114M pumps, and 340 organizer, was used for the second-column chromatography. The eluent was monitored at 230 nm in a 1-cm light path with a Gilson Holochrome UV monitor, connected to a Linear recorder, Model 261/MM.

Performance of tandem HPLC

The tandem HPLC assembly is illustrated in Fig. 1. An anion-exchange Spherogel-TSK IEX-540 DEAE column (C1) (System 1) is connected in tandem with a reversed-phase Ultrasphere ODS column (C2) (System 2) through a tee tube (T). In each system, programmed elution is performed with a 421 CRT controller (Controllers 1 and 2). Controller 1 regulates two pumps (P1 and P2) of System 1 to perform a series of stepwise elutions, synchronized with Controller 2. Buffers B1 (0.02 *M* Tris-acetic acid, pH 8.0) and B2 (0.4 *M* ammonium hydroxide–methanesulfonic acid–0.02 *M* Tris-acetic acid, pH 8.0), pumped by P1 and P2 respectively, are combined in a mixing chamber (M1) and flow into the anion-exchange column (C1) after passing through the sample injector (SI). Controller 2 regulates the flow-rates of two pumps (P3 and P4) in System 2 to perform a repetitive linear gradient elution, syn-

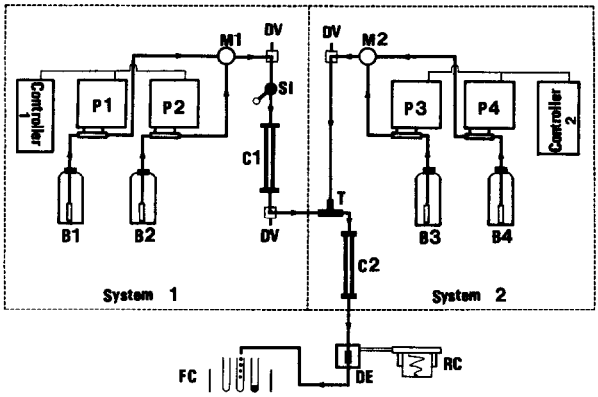

Fig. 1. Schematic diagram of the tandem HPLC system for peptide mapping of very large proteins. The assemblies are explained in the text. Symbols not indicated in the text are: DV = drain valve; FC = fraction collector; DE = wavelength-tunable UV detector; RE = recorder.

chronized with System 1. Buffers B3 (0.1% TFA) and B4 (acetonitrile containing 0.1% TFA), pumped by P3 and P4 respectively, are combined in a mixing chamber (M2) and flow into the reversed-phase column (C2) through the tee tube after the stepwise elution from the column (C1) is stopped.

For the first chromatography a peptide mixture is applied in System 1 to the anion-exchange column (C1) for 15 min at a flow-rate of 0.5 ml/min only with buffer B1. At this step, P2 does not pump buffer B2 (0% B2). After the eluate from C1 is applied directly into the reversed-phase column C2, System 1 is stopped; simultaneously, the second chromatography begins as System 2 starts pumping at a flow-rate of 1.0 ml/min with a linear gradient from 0 to 45% of acetonitrile during 60 min. Column C2 is equilibrated again with B3 for 5 min after the linear gradient elution is finished; then the flow in System 2 is stopped. By this step, the first cycle of the tandem chromatography is completed. Then, System 1 for the first chromatography starts again to elute peptides stepwise from the anion-exchange column (C1) with 5% B2. After the application of the eluate to C2, the second chromatography is repeated exactly as described above. The pumping ratio of B2 for the first chromatography is increased in a stepwise manner as follows: 0, 5, 10, 15, 20, 25, 30, 35, 40, 50 and 100% B2. The stepwise elution for anion-exchange chromatography and the linear gradient elution for reversed-phase chromatography are synchronized by computer programs of Controllers 1 and 2.

Identification and nomenclature of peptides

The peptides were identified by their amino acid composition. Amino acid analysis was performed with the Beckman amino acid analyzer (Model 121M)[14]. Tryptic peptides of intact human ceruloplasmin are given the prefix T and are numbered consecutively in their order in the sequence[13,14].

RESULTS AND DISCUSSION

Separation of the tryptic peptides of human ceruloplasmin by automated tandem HPLC

Human ceruloplasmin consists of a single polypeptide chain and is the second

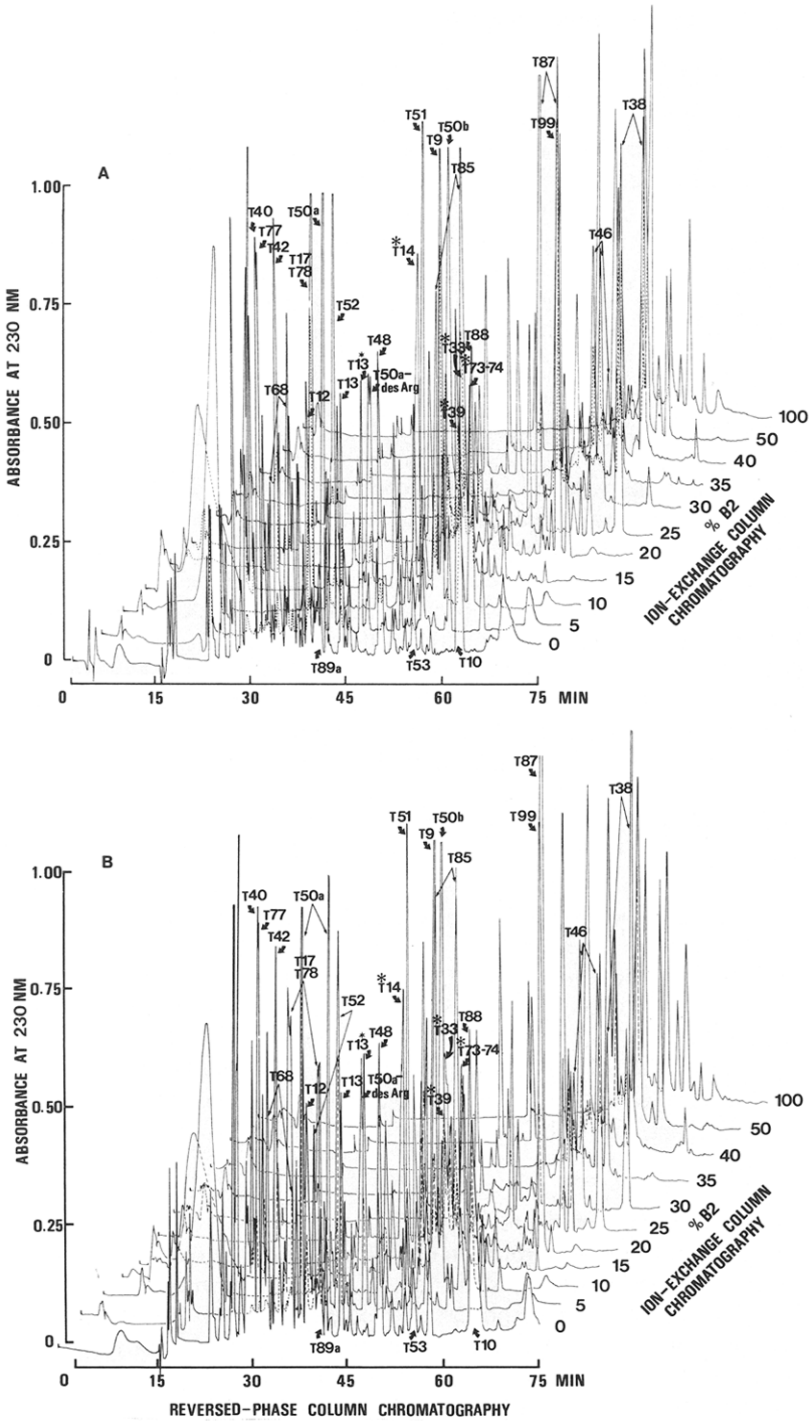

Fig. 2. A three-dimensional visualization of the automated tandem chromatogram. A and B are two separate experiments that illustrate the reproducibility of the method. The nomenclature for peptides described in Experimental is used to illustrate the elution positions of some of the tryptic peptides identified. Peptides identified as two peaks are shown by two fine arrows and peptides identified as a single peak by a thick arrow. Glycopeptides are indicated by an asterisk. Peptide T13* marked with a star was an unexpected peptide obtained due to cleavage of an Arg–Pro bond in peptide T13 with trypsin.

largest plasma protein for which the complete amino acid sequence has been determined[1]. The protein contains 1046 amino acids and has a potential for yielding 103 tryptic peptides[13,14]. The tryptic digest of the carboxymethylated single-chain ceruloplasmin was separated by the automated tandem HPLC system. Although a long, continuous chromatogram is obtained, the profile is shown in Fig. 2A in three-dimensional visualization. Each horizontal profile represents one cycle in the chromatography, which consists of stepwise elution from the anion-exchange column, application of the eluent to the reversed-phase column, and linear gradient elution from the reversed-phase column. In each horizontal profile the first 15 min indicate stepwise elution in System 1, and the remaining 60 min show linear gradient elution in System 2. Because the reversed-phase column is equilibrated with 0.1% TFA at each step after linear gradient elution, a pre-chromatography time of 5 min is drawn before time 0 in Fig. 2. The concentration (% B2) of B2 buffer for the stepwise elutions of the anion-exchange column is shown at the right side of each horizontal profile.

When the program described is applied to ceruloplasmin, *ca.* 260 peaks are obtained, compared with the expected 100 theoretical tryptic peptides. This demonstrates the excellent resolution of the method. Of course, the number of peaks exceeds the expected 100 theoretical peptides, because some peptides are duplicated in the adjacent stepwise elutions, and also because of incomplete cleavage. In order to evaluate the efficiency as a separation method of peptides, many peaks selected randomly were analyzed by amino acid analysis. At least 70% of the peaks analyzed contained pure peptides that were identified in the sequence of ceruloplasmin. Although some of the peaks contained two peptides, they could be also identified in the sequence. Some of the peaks identified are indicated in Fig. 2.

Reproducibility of the method

The reproducibility of the method is demonstrated in Fig. 2. The profiles were obtained by repeating the chromatography of the same digest of the single-chain ceruloplasmin. To facilitate the comparison of the two chromatograms, many of the major peaks are identified by the number of the tryptic peptide to which they correspond. It is evident that the patterns of the two chromatograms are reasonably reproducible in spite of the complexity of the peptide mixture analyzed. To be sure, some peptides, *e.g.* T50a and T52 in Fig. 2A, are obtained as two peaks in Fig. 2B; conversely, T87, T17 and T78 in Fig. 2B are obtained as two peaks in Fig. 2A, and so forth. However, as illustrated later, it is possible to identify the duplicated peaks in adjacent profiles by comparing their retention time in reversed-phase chromatography.

Recovery of the glycopeptides of ceruloplasmin

Human ceruloplasmin is a glycoprotein exhibiting four sites of attachment for glucosamine oligosaccharide, which we identified by determination of the complete amino acid sequence[14]. Earlier, Ryden and Eaker[15] expended much effort in isolating the glycopeptides for three sites by conventional methods, but could not isolate the fourth glycopeptide because of the complexity of the peptide digest. By a combination of gel filtration and reversed-phase chromatography[7] we were able to isolate all four glycopeptides in good yield, but the manual procedure was lengthy. However, all

four glycopeptides were easily purified from the tryptic digest of the whole ceruloplasmin molecule in 16 h by use of our automated tandem HPLC system. The yields of the four glycopeptides T14, T33, T39, and T73–74 were 53, 32, 55, and 40% respectively; these values are higher than those we obtained earlier by a manual combination of gel filtration and reversed-phase chromatography[7]. With the automated system, peptides T14 and T73–74 were obtained as several peaks in reversed-phase chromatography. Multiple forms of these peptides were also observed previously[7] and are probably due to the heterogeneity of the carbohydrate structure and/or deamidation of glutamine.

Human ceruloplasmin exists in two different forms, Type I and Type II; these can be separated by hydroxylapatite chromatography; the only difference between them is believed to be that Type I is glycosylated at position Asn-339 in tryptic peptide T33, whereas Type II is not[7,13]. Because the ceruloplasmin preparation was a mixture containing *ca.* 85% of Type I and 15% of Type II, the non-glycosylated form of peptide T33 should be present in only small amounts. As yet, this minor peptide has not been isolated, but it may be present in one of many small peaks not yet analyzed. However, the method has proved to be effective for purifying all of the major glycosylated peptides from the very complex mixture of ceruloplasmin peptides by a single operation of the chromatography system.

Comparison of the peptide maps of the single-chain and fragmented forms of ceruloplasmin

In order to determine whether the automated tandem HPLC system is applicable to the identification of differences between the single-chain and the fragmented (autolytically degraded) forms of ceruloplasmin, the tryptic digests of both forms were analyzed by the automated tandem HPLC system. The same peptides would be expected for the intact and the degraded forms of ceruloplasmin, except at the junction of the 67-kDa and 50-kDa fragments, and likewise at the junction of the 50-kDa and 19-kDa fragments. In each case, the plasmin-like cleavage that forms the fragments is followed by action of a carboxypeptidase B that removes the C-terminal basic amino acid. Thus, in the case of the 67-kDa fragment, the C-terminal peptide (designated T50a) should end with Arg-481. In fact, single-chain ceruloplasmin does yield T50a, but the corresponding peptide from the 67-kDa fragment lacks Arg-481 and is therefore designated T50a-desArg (Fig. 3). Similarly, intact ceruloplasmin yields peptide T89a, which ends with Lys-887, but the 50-kDa fragment yields T89a-desLys. As an example, the peaks containing peptides T50a and T50a-desArg are shown in Fig. 4, for which only the essential profiles have been selected from the chromatograms.

Although peptides T50a and T50a-desArg were obtained from both the single-chain and the degraded forms of ceruloplasmin, the ratio of the yields of T50a to T50a-desArg was *ca.* 9:1 for the single-chain form (Fig. 4A) and *ca.* 1:4 for the

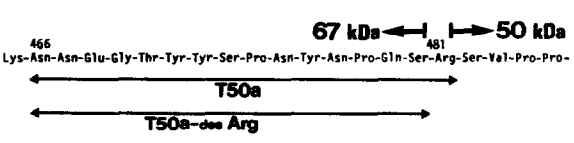

Fig. 3. Amino acid sequence at the junction between the 67-kDa and 50-kDa fragments.

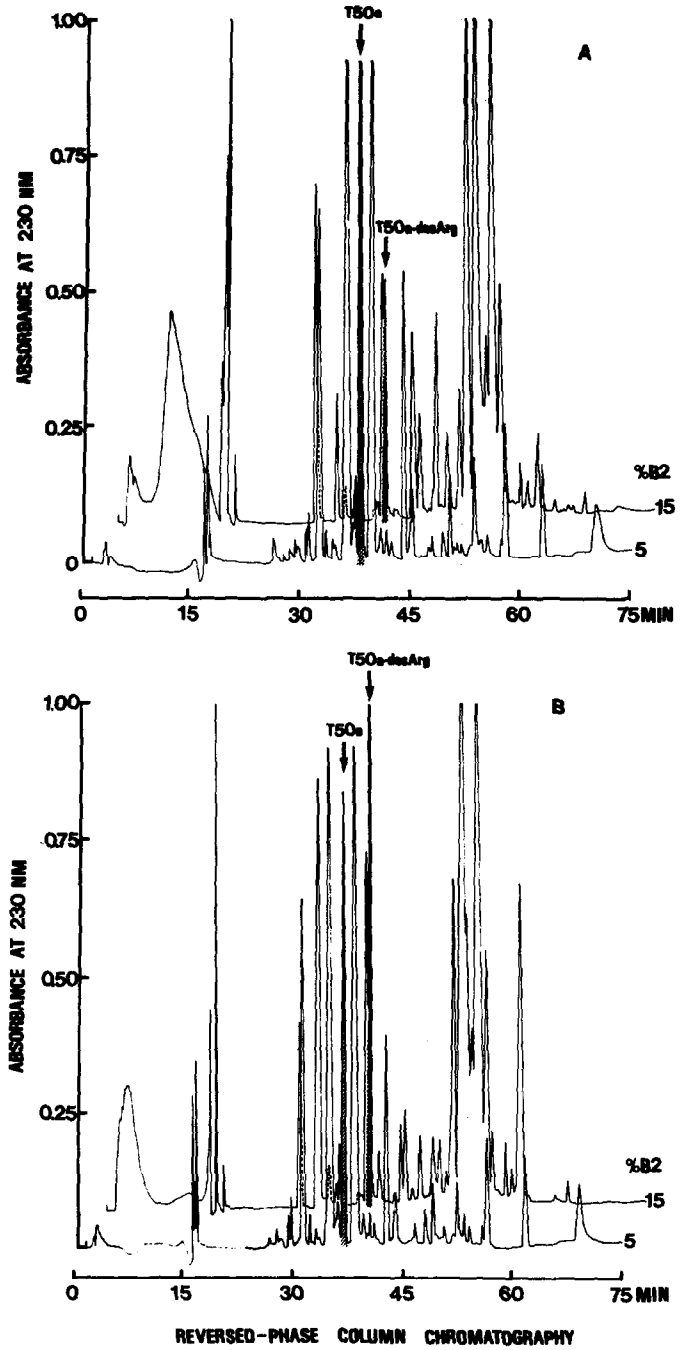

Fig. 4. Partial comparison of the peptide maps between the single-chain form (A) and degraded form (B) of ceruloplasmin. The peaks containing T50a and T50a-desArg are shaded. The concentration (% B2) of B2 buffer for the stepwise elutions of the anion-exchange column is shown at the right side of each horizontal profile. In Fig. 4A the absorbance for T50a and the other main peaks was off scale, and similarly for T50a-desArg in Fig. 4B.

degraded form (Fig. 4B). Peptide T50a was eluted from the anion-exchange column at 5% B2. Because peptide T50a-desArg is more acidic than T50a, it was eluted two cycles later than T50a, *i.e.* at 15% B2. However, the peptides had almost identical retention times in the reversed-phase column chromatography (T50a, 38.4 min; T50a-desArg, 38.7 min). The ratio of the peptides recovered may reflect the fact that the ceruloplasmin molecule rapidly undergoes spontaneous proteolytic cleavage. Thus, it is very difficult to obtain 100% single-chain form. Furthermore, preparations of the degraded form always contain a small amount of the undegraded form. These results indicate that our method is also effective for detecting the molecular heterogeneity of very large proteins, such as that due to proteolytic modification of ceruloplasmin.

Limitations of the method

An anion-exchange column was selected for the first separation, because human ceruloplasmin is an acidic protein (pI = 4.4) from which many acidic peptides would be expected. In order to estimate the distribution of charge and hydrophobicity of all of the tryptic peptides expected from a completely specific cleavage of the protein, the charges of the theoretical peptides are plotted against ln(1 + H) in Fig. 5, in which H indicates the hydrophobicity of the peptides, calculated by the method of Sasagawa *et al.*[16] using their list of non-weighted retention constants. The charges of the peptides are estimated from the constituent amino acids of the peptide at pH 8.0 by the microcomputer method of Manabe[17]. The charges of the peptides of ceruloplasmin range from +2 to −5, and the values of ln(1 + H) range from −2.3 to 6.1. All of the theoretical tryptic peptides are represented by solid circles in Fig. 5, and those that were identified by amino acid composition are denoted by T symbols,

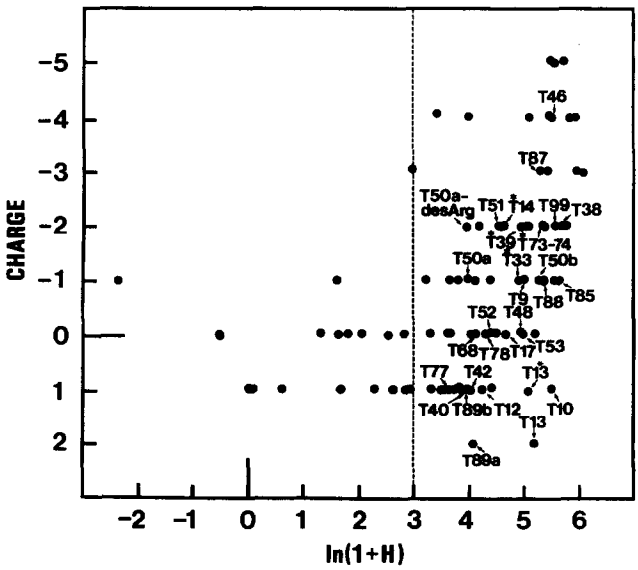

Fig. 5. Distribution between charge and ln(1 + H) of the tryptic peptides of human ceruloplasmin. The peptides identified are designated as described in Experimental. Glycopeptides are indicated by an asterisk. A dotted line is drawn at ln(1 + H) = 3.

as in Fig. 2. The distribution of the peaks of the actual chromatogram is shown in Fig. 6. In this figure the vertical scale indicates the % B2 of the stepwise elution in anion-exchange chromatography, and the horizontal scale indicates the retention time in the reversed-phase chromatography. The peptides identified are also indicated in Fig. 6. By comparing Figs. 5 and 6 it can be seen that peptides eluted from the anion-exchange column without interaction at 0% B2 generally have a positive charge or zero net charge; *e.g.*, T10, T13, T40, T42, T48, T77, etc. This is true even for some peptides that were eluted as duplicated peaks in the successive elutions at 0 and 5% B2; *e.g.*, T68, T52, etc. In contrast, peptides that were identified and which had a negative charge (and some with zero charge) interacted with the anion-exchange column; *e.g.*, T9, T88, T85, T50b, T39, T33, T14, T87, T46, T38, etc. (Figs. 5 and 6).

The peptides with the lowest charge (-5) have not yet been identified. However, peptide T46, with a charge of -4, was eluted at both 30 and 35% B2 (Fig. 6). The unidentified peaks eluted at 40, 50 and 100% B2 probably contain the peptides with the highest negative charge. In order to be sure that no peptides remain bound to the column, it should be washed with 1 *M* ammonium hydroxide–methanesulfonic acid–0.02 *M* Tris-acetic acid (pH 8.0) before application of the next sample.

The retention time in reversed-phase chromatography[16] is linearly related to the natural logarithm of the sum of the retention constants. All peptides that were identified by amino acid composition had a value of ln(1 + *H*) greater than 3, and

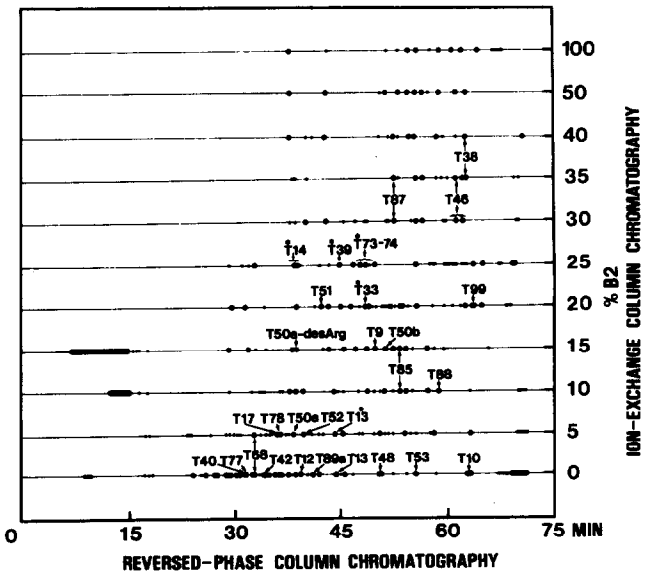

Fig. 6. Distribution of all of the peaks of the actual chromatogram shown in Fig. 2A. Major peaks that have heights greater than 0.1 on the absorbance scale at 230 nm are indicated by solid circles, and minor peaks that have less than 0.1 are indicated by dots. The peptides of Fig. 2A that were identified by amino acid composition are denoted by symbols. Peptides duplicated in the adjacent stepwise elutions are shown by two arrows; *e.g.* T68, T85, T87, T46 and T38. Peptides obtained as multiple peaks in the reversed-phase chromatography are indicated by a bracket. Glycopeptides are denoted by an asterisk over the T symbol.

thus they were separated by the reversed-phase chromatography (see right side of Fig. 5). In contrast, components with a value of $\ln(1 + H)$ less than 3 will probably not be separated by reversed-phase chromatography. Such components include many di-, tri-, and tetrapeptides and the free amino acids arginine and lysine, which can be expected from tryptic digestion of many proteins. In the case of ceruloplasmin, the small hydrophilic peptides were not separated and were obtained as several broad peaks during chromatography on the anion-exchange column (Figs. 2 and 6). Of the peptides that were identified, those with the highest hydrophobicity were T38 and T85; both have a value of $\ln(1 + H) = 5.7$. The peptide with the highest value (6.1) has not yet been identified, but there are many peaks with longer retention times than T38 and T85 (Fig. 6), which are not yet analyzed. Of course, some of the peptides with high hydrophobicity may be present in the insoluble material which was removed by centrifugation before chromatography. If a large amount of precipitate is formed during enzymatic digestion, a non-ionic denaturing reagent, such as urea or non-ionic detergent, may be included in the buffer for the chromatography, because these non-volatile materials can be removed by the second chromatography.

Other applications of the method

Because of the small amount of sample required (as little as 10 nmol) and because of its flexibility, the method described should be very useful for peptide mapping of genetic and post-translational variants of large proteins for which the amino acid sequence is already known. We plan to use the method to compare the HPLC peptide maps of normal ceruloplasmin and the ceruloplasmin of individual patients with Wilson's disease. Another application will be to identify the sites of substitution in known genetic variants of human serum albumin. The method will be particularly useful in our current study of the deletions in the structure of immunoglobulins that result in "heavy-chain disease" proteins and also for identification of the allotype differences in human immunoglobulins. The method is also useful in the strategy for protein sequencing. For example, we have applied it successfully to separate a digest of leucine-rich α_2-glycoprotein with dilute acid in order to obtain an overlapping peptide for a segment of sequence that could not be completed by conventional methods[18].

CONCLUSIONS

We have developed an automated tandem HPLC system for peptide mapping of very large proteins in order to analyze molecular heterogeneities as well as to purify peptides efficiently. The method was applied to the analysis of a tryptic digest of human ceruloplasmin, and the digest was resolved reproducibly into *ca.* 260 peaks within 16 h. The number of peaks exceeds the expected 103 theoretical peptides, because some peptides are duplicated in the adjacent stepwise elutions and also because of incomplete and/or non-specific cleavages. The larger a protein is, the more complex the peptide mixture will become because of incomplete and non-specific cleavage, and thus the separation of the peptides will become more difficult. However, of the peptide peaks thus far analyzed for amino acid composition, two-thirds were pure and could be identified after a single operation of the chromatography system. This includes all four glycopeptides. Comparison of the peptide maps of the single-

chain and degraded forms of ceruloplasmin indicates that the method was also effective for detecting the molecular heterogeneity of a very large protein due to proteolytic modification.

The high resolution, reproducibility, speed, and recovery of a wide range of peptides are all important factors for comparative peptide mapping of very large proteins that exhibit molecular microheterogeneity, such as carbohydrate or genetic variants. The method described seems to possess the desired features, and is very flexible. The column for the first chromatography can be changed to a cation-exchange column. If the amount of protein is small, both columns can be changed to smaller columns. The programs for both dimensions also can be changed, depending on how complex the peptide mixture is, etc. Because the method is very powerful and flexible in the purification of many peptides from a very complex peptide mixture by chromatography in a single operation, it may be a complementary tool in looking for peptide probe sequences or for sequence support in a strategy for DNA sequencing and, likewise, for the preparative purification of peptides in the strategy for protein sequencing of very large proteins.

ACKNOWLEDGEMENTS

We thank S. A. Dorwin, K. Huss, and J. Madison for their excellent technical assistance. This work was supported by grants from the NIH (AM19221 and CA08497) and from the American Cancer Society (IM-21).

REFERENCES

1 F. W. Putnam (Editor), *The Plasma Proteins*, Vol. 4, Academic Press, Orlando, FL, 1984, 2nd ed., p. 1.
2 N. L. Anderson, R. P. Tracy and N. G. Anderson, in F. W. Putnam (Editor), *The Plasma Proteins*, Vol. 4, Academic Press, Orlando, FL, 1984, 2nd ed., p. 221.
3 M. T. W. Hearn, F. E. Regnier and C. T. Wehr (Editors), *High-Performance Liquid Chromatography of Proteins and Peptides*, Academic Press, New York, 1983, p. 1.
4 M. Elzinga (Editor), *Methods in Protein Sequence Analysis*, Humana Press, Clifton, NJ, 1982, p. 1.
5 F. W. Dwulet and M. D. Benson, *Proc. Nat. Acad. Sci. U.S.*, 81 (1984) 694.
6 V. J. Lewis, R. N. P. Singh, L. J. Lewis, B. K. Seavey and Y. N. Sinha, *Proc. Nat. Acad. Sci. U.S.*, 81 (1984) 385.
7 N. Takahashi, Y. Takahashi, T. L. Ortel, J. N. Lozier, N. Ishioka and F. W. Putnam, *J. Chromatogr.*, 317 (1984) 11.
8 N. Takahashi, Y. Takahashi and F. W. Putnam, *J. Chromatogr.*, 266 (1983) 511.
9 H. Kratzin, C.-Y. Yang, J. U. Krushe and N. Hilschmann, *Hoppe-Seyler's Z. Physiol. Chem.*, 361 (1980) 1591.
10 H. P. J. Bennett, C. A. Browne and S. Solomon, *J. Liq. Chromatogr.*, 3 (1980) 1353.
11 M. Noyer, F. E. Dwulet, Y. L. Hao and F. W. Putnam, *Anal. Biochem.*, 102 (1980) 450.
12 I. B. Kingston, B. L. Kingston and F. W. Putnam, *Proc. Nat. Acad. Sci. U.S.*, 74 (1977) 5377.
13 T. L. Ortel, *Dissertation*, Indiana University, Bloomington, IN, 1983 (available from University Microfilms International, 300 North Zeeb Road, Ann Arbor, MI 48106).
14 N. Takahashi, T. L. Ortel and F. W. Putnam, *Proc. Nat. Acad. Sci. U.S.*, 81 (1984) 390.
15 L. Ryden and D. Eaker, *Eur. J. Biochem.*, 44 (1974) 171.
16 T. Sasagawa, T. Okuyama and D. C. Teller, *J. Chromatogr.*, 240 (1982) 329.
17 T. Manabe, *Kagaku no Ryoiki*, 36 (1982) 470 (in Japanese).
18 N. Takahashi, Y. Takahashi and F. W. Putnam, *Proc. Nat. Acad. Sci. U.S.*, 82 (1985) 1906.