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COVALENT CHROMATOGRAPHY AS A MEANS OF ISOLATING THIOL PEPTIDES FROM LARGE PROTEINS

APPLICATION TO HUMAN CERULOPLASMIN

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SUMMARY

The use of activated thiopropyl-Sepharose for simple and rapid isolation of thiol peptides from large proteins was investigated using ceruloplasmin (a copper protein of molecular weight 134,000 containing three cysteines and six disulphides) as a test case. Optimal results for the immobilization of the protein to the activated gel were obtained at pH 4.0 in the presence of 8 *M* urea and 0.05 *M* ethylenediaminetetraacetic acid. In these conditions 96% of the protein thiol groups were attached to the adsorbent.

The immobilized protein was digested with either pepsin or trypsin. The liberated non-thiol peptides were eluted from the gel together with the protease after the digestion. After washing, the covalently attached thiol peptides were eluted in reducing buffer, desalted on the hydrophobic gel Sephadex LH-20 and carboxymethylated. The peptides were purified in a two-step procedure involving gel filtration on Sephadex G-25 and either column electrophoresis or ion-exchange chromatography.

The two sets of peptides were derived from four different regions in the protein. They were 12–39 residues in length and together accounted for 152 residues. It is shown that the peptide chain was susceptible to proteolytic attack also close to the point of attachment (two residues away). One peptide with two thiol groups proved to be derived from an area containing one disulphide bridge in addition to cysteine. This bridge could be identified in a separate experiment where a second enzyme was used to release the disulphide peptide after the first digestion and washings.

INTRODUCTION

In a project concerned with the molecular evolution of the blue copper-containing proteins we have been interested to compare amino acid sequences around the cysteinyl residues in these proteins. We have used covalent chromatography[1] as a rapid and simple technique for obtaining cysteinyl peptides in high yield. This term implies a covalent attachment of cysteine side chains to a matrix containing activated thiol groups by means of thiol–disulphide exchange. The activation of the matrix thiol group with 2-pyridyldisulphide[1]

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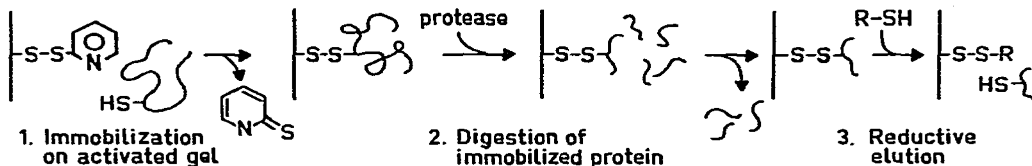
has several advantages. In the immobilization reaction

the thiopyridone formed is an excellent leaving group, which promotes quantitative reaction, and furthermore can be followed by its absorption at 343 nm. The use of this technique for thiol peptide isolation was first described in a report from this laboratory in 1975[2]. Since then a number of modifications and extensions have been introduced and the conditions of the various steps have been optimized. We want to report these results below.

Two different variations of the technique are possible (Fig. 1). The first involves the attachment of the protein to the matrix and subsequent proteolytic degradation. In the second, suggested by Svenson and co-workers[3], the protein itself is reacted with pyridyldisulphide to activate its thiol groups *in situ*. Thereafter the derivatized protein is digested with protease and the digest reacted with a matrix containing thiol groups to trap the activated peptides. A drawback with the latter approach is that the thiol gel itself is reducing and might promote a premature elution of the adsorbed peptides by forming an internal disulphide bridge, at least if the degree of substitution is high enough. The first approach might have the effect of not allowing several cysteine thiol groups on one peptide chain to be immobilized simultaneously. This problem was, however, not encountered with ceruloplasmin. In our work we have used the first approach exclusively.

Part of the results described here have appeared in a symposium report[4].



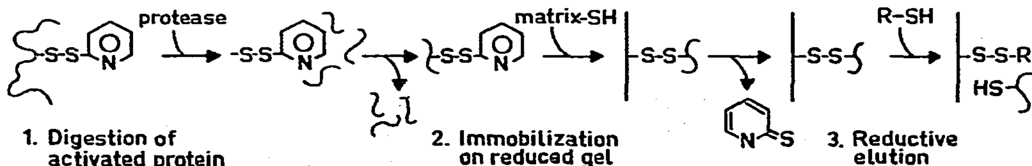


Fig. 1. Strategies for obtaining thiol peptides by covalent chromatography.

MATERIALS AND METHODS

Materials

Thiopropyl-Sepharose. The thiopropyl-Sepharose 6B (2-thiopropyldisulphide hydroxypropyl ether agarose) was obtained from Pharmacia, Uppsala, Sweden. It contained 416 μmoles of active structures per gram dry weight, corresponding to approximately 20 μmoles of active structures per ml of sedimented gel bed. Low-molecular-weight thiol (2-mercaptoethanol) released 29.5 μmoles of thiopyridone per gram of washed activated gel, dried by aspiration on a glass funnel.

Ceruloplasmin. Ceruloplasmin was obtained from AB Kabi (Stockholm, Sweden) and further purified by gel filtration on elutriated Sephadex G-150 to get an enzyme with an A_{610}/A_{280} ratio of 0.043–0.044, indicating >95% pure enzyme[5]. The concentrations were estimated from 280-nm absorption using $A_{280}^{1\%;\,1\,cm} = 15.3$. The samples used were known to be slightly proteolytically nicked[6]. This was, however, not expected to disturb the experiments.

Other chemicals. 2-Thiopyridyldisulphide was obtained from Aldrich (Beerse, Belgium). Trypsin was a TPCK-treated enzyme from Sigma (St. Louis, MO, U.S.A.). Pepsin was three times crystallized enzyme from Serva (Heidelberg, G.F.R.). Other chemicals were of analytical grade.

Analytical methods

Reaction with dithiopyridyldisulphide. The extent of the reaction of ceruloplasmin with dithiopyridyldisulphide or the activated gel was estimated from the absorption of liberated 2-thiopyridone at 343 nm, assuming a molar absorptivity $\varepsilon_{343} = 8108 \cdot 10^3\ M^{-1}\ cm^{-1}$ (ref. 7). A spectrum was taken routinely to allow baseline correction by linear extrapolation from higher wavelengths. This is essential for a quantitative estimation of the immobilization of protein, when one measures the supernatant of the gel suspension, where scattered particles are often present.

To estimate the amount of bound protein (not necessarily the same as bound thiols) an aliquot of the supernatant was desalted on a small column of Sephadex G-25 (disposable column PD-10, Pharmacia) and the absorption of the pooled protein peak was measured at 280 nm. The desalting was necessary to remove thiopyridone which has a second absorption band at 270 nm.

Preparative methods

Immobilization of protein. A selected amount of lyophilized Thiopropyl-Sepharose was rinsed with water on a glass funnel, dried by aspiration and transferred to a tarred centrifuge tube and weighed. Stock solutions of buffers, solid urea or guanidine HCl and water was added to achieve the desired concentrations. The buffers used were sodium formate (pH 3.0 and 4.0), sodium acetate (pH 4.5 and 5.0) and Tris · HCl (pH 8.0). Concentrations of buffers were 0.2 *M*. In some experiments nitrogen was bubbled through for 30 min. Finally the protein was added as a concentrated solution (about 5%). The final volume was about 10 ml per g of lyophilized powder (2.5 g gel dried by aspiration). The mixture was incubated at room temperature by end-over-end rotation for a prescribed time, which in the standard procedure was 5 h. The suspension was finally centrifuged and the spectrum of the supernatant was measured for an estimation of the extent of reaction.

Digestion of immobilized protein. The gel with attached protein was transferred to a glass filter funnel and washed with water and several volumes of digestion buffer. The pH 2 buffer used for peptic digestions was 1.3 *M* acetic acid, 0.54 *M* formic acid. The pH 8.0 buffer for tryptic digestions was 0.1 *M* ammonium bicarbonate. The gel was transferred to a new centrifuge tube and buffer added to get the same volume as in the immobilization or slightly less. An appropriate amount of enzyme dissolved in 1 m*M* HCl was added to get an enzyme-to-substrate ratio of about 1:25. Incubations were continued at room temperature by end-over-end rotation overnight (pepsin) or for 4 h (trypsin). In some experiments, portions of enzyme twice as large were used.

Elution of non-adsorbed peptides. After the digestion the gel was transferred anew to the glass filter funnel and rinsed with digestion buffer followed by the same buffer containing 1 *M* NaCl and finally with 0.1 *M* Tris · HCl, pH 8.0. The gel was then packed in a small column and eluted with 0.1 *M* Tris buffer containing 0.05 *M* 2-mercaptoethanol at a rate of about 2 cm/h. The eluate was collected and read at 280 nm and a higher wavelength (400 nm) to obtain a convenient range of absorption values. The peak containing eluted thiol peptides and thiopyridone was collected.

Fractionation of peptides. Prior to fractionation, the peptides were carboxymethylated with iodoacetic acid. The fractionations involved a Sephadex G-25 gel filtration to obtain a size separation and in some experiments removal of thiopyridone and other reagents. The further fractionation with column zone electrophoresis of peptic peptides have been described in connection with the structure determination of these peptides[8]. The tryptic peptides were fractionated by gradient chromatography on SP-Sephadex C-25 cation exchanger using a gradient (0.02 to 0.25 *M*) of ammonium acetate buffer pH 5.0 for elution. Analysis of the eluate at 230 nm showed the gradient (acetate absorbs at this wavelength) as well as the peptide peaks superimposed. A similar procedure was used for the separation of peptides by gradient chromatography on QAE-Sephadex A-25, where ammonium acetate buffer pH 8.5 (0.02 to 0.5 *M*) was used for elution.

The amino acid analysis of peptides were done as described previously[8].

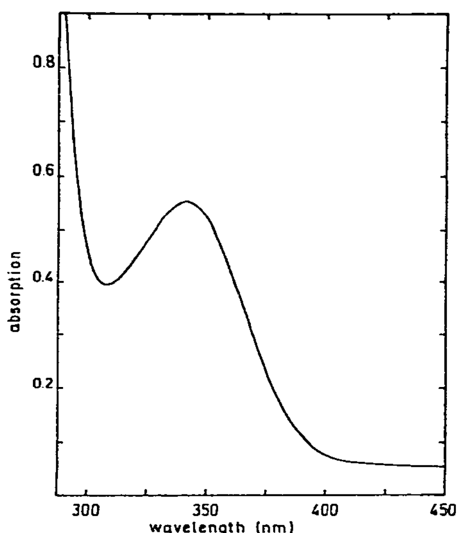
RESULTS

Experiments on an analytical scale

Reactions of ceruloplasmin with 2-thiopyridyldisulphide. A 5-mg portion (0.038 μmoles of protein or 0.114 μmoles of thiol) was incubated with 1 ml of 4 m*M* solution of 2-pyridyldisulphide for 3 h at room temperature. The reactions were carried out at pH 3.0, 4.0, 4.5, 5.0 and 8.0 in presence of 8 *M* urea with and without 0.05 *M* Na_2EDTA and with and without deaeration with nitrogen.

In the absence of chelator (EDTA) there was no reaction at all. In the presence of EDTA the extent of the reaction was identical at the different Ph values as measured from the 343 nm absorption. The minimum in the spectrum at 310 nm was, however, much deeper at pH 4 than at the other pH values, indicating less side reactions in particular as compared to pH 3.0 and 8.0. Deaeration had only a slight effect on the reaction yield, which was close to 93% in all cases. Thus the pH 4.0 buffer was used for the subsequent immobilization reactions.

Immobilization of ceruloplasmin on Thiopropyl-Sepharose. A 5-mg portion (0.038 μmoles of protein or 0.114 μmoles of thiol) was incubated with 350 mg gel



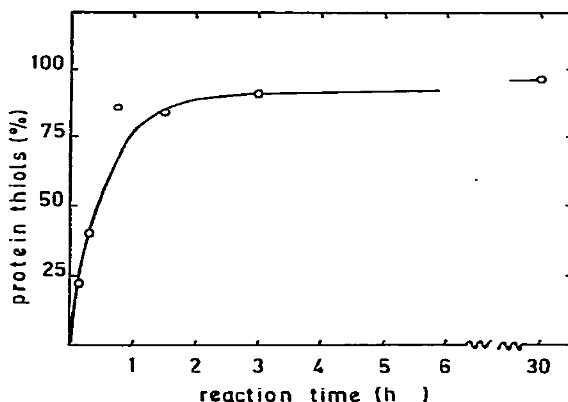


Fig. 2. Spectrum of the supernatant after incubation of 300 mg of human ceruloplasmin with 18 g of thiopropyl-Sepharose dried by aspiration after 5 h.

Fig. 3. Time course of the reaction of 5 mg of human ceruloplasmin with 300 mg of thiopropyl-Sepharose dried by aspiration. The percentage of total protein thiols (0.114 μmoles) reacted as judged by amount of released thiopyridone is indicated on the abscissa.

dried by aspiration at pH 4.0 and in presence of 8 M urea and 0.05 M Na_2EDTA. The final volume was 1.5 ml. A typical spectrum obtained on the immobilization mixture is shown in Fig. 2.

The amount of protein reacted at different times is shown in Fig. 3. The reaction is nearly complete after 2 h. A reaction time of 5 h was used for subsequent experiments.

The capacity of the gel was studied in a different set of experiments. In the analytical scale (5–20 mg of protein) about 60% of added protein was reacted when up to 2% of the active structures on the gel was used. This result was not consistent with the large-scale experiments (1 μmole or more of protein) where the extent of reaction was typically 95% or more. The reason for this difference is not known. The extent of reaction was the same if the amount of non-reacted protein was estimated, which means that the protein molecules had all reacted with three thiol groups.

Since a background was obtained in the blank trials a separate series of experiments were run to study the release of thiopyridone from the incubation with no protein present. In the typical conditions of 5 h at pH 4.0 in the presence of 8 M urea about 0.2% of the active structures was liberated as thiopyridone. The continued release was linear in time and amounted to about 0.5% per 50 h (Fig. 4). It was considerably larger when guanidine HCl (spectroscopic grade) was used instead of urea. The released thiopyridone was retarded on a PD-10 column and thus was presumably of low molecular weight.

Experiments on a preparative scale

Preparation of thiol peptides. In the preparative experiments 1–3 μmoles (130–

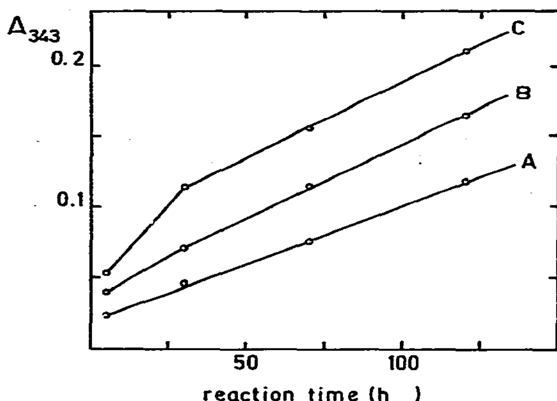


Fig. 4. Release of thiopyridone in the supernatant after incubation of 250 mg of thiopropyl-Sepharose dried by aspiration in (A) water, (B) 0.2 *M* sodium formate pH 4.0 with or without 8 *M* urea and (C) 0.2 *M* sodium formate pH 4.0 with 6 *M* guanidine HCl. An absorption of 0.2 at 343 nm corresponds to 1 % of the total thiopyridyl structures on the gel.

400 mg) of ceruloplasmin were incubated with thiopropyl-Sepharose as described in the *Preparative methods* section using the pH 4.0 buffer in 8 *M* urea and 0.05 *M* Na_2EDTA; 3–4 g of lyophilized gel (corresponding to 5.5–7.5 g of gel dried by aspiration or 10–15 ml settled gel bed) was used per μmole of protein. The extent of the reaction was about 95 %. The digestions were carried out as described in the *Preparative methods* section and followed by washings and transfer to pH 8.0 buffer for reductive elution. This was best done in a column by slow continuous pumping of the mercaptoethanol-containing buffer. The eluted thiopyridone and peptides were then obtained in about 20 ml of eluate for a 10-ml column.

Derivatization of thiol peptides. The eluate from the thiopropyl column contained about a 100-fold molar excess of thiopyridone over peptide. Still it could be lyophilized and redissolved in about 5 ml of water and adjusted to pH 8.0 by addition of solid Tris. The alkylation of this mixture with iodoacetic acid was not completely satisfactory. Thiopyridone apparently consumed the alkylating agent since the 343-nm band disappeared and a new component with absorption maximum at 288 nm was formed. The first order rate constant of this reaction was estimated to be 1.8 M^{-1} min^{-1}.

Although this presumably is slower than the carboxymethylation of cysteine[9], the addition of only a slight molar excess of alkylating agent still produced peptides that apparently were dissimilar only in the cysteine according to their amino acid composition which indicates non-quantitative carboxymethylation. This approach was therefore abandoned. Instead we fractionated the eluate from the thiopyridone column on a second column of the slightly hydrophobic gel Sephadex LH-20 at acid pH (1 m*M* HCl) where no oxidation of thiol groups was expected. In this step (Fig. 5) the peptides, being charged, elute in the void fraction regardless of size, while the buffer salts, thiopyridone and excess mercaptoethanol and its dimer are retarded. The peptide fractions were pooled and lyophilized to get an easily dissolved powder. Alkylation could then be performed in the standard way under controlled conditions[10]. Excess reagents were removed anew by chromatography on a small Sephadex LH-20 column.

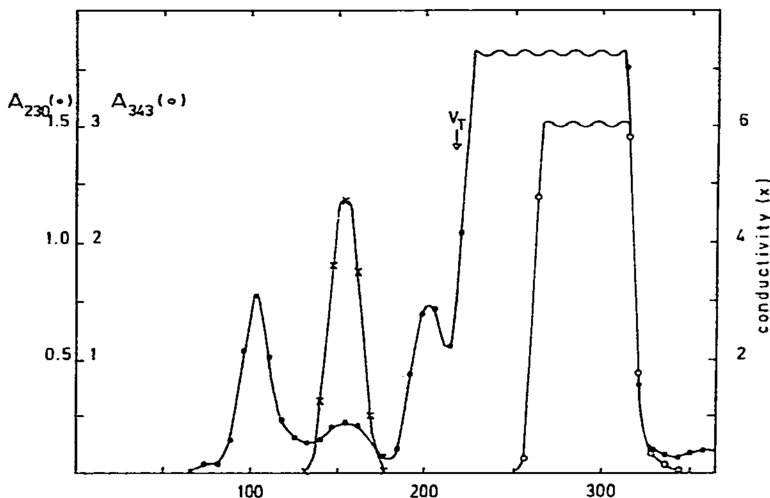


Fig. 5. Chromatography of 20 ml of eluate from the reductive elution of an 11-ml bed of thiopropyl-Sepharose with coupled thiol peptides on a column (3.2 × 27 cm) of Sepharose LH-20. Fractions of 7.3 ml were collected and analyzed for absorption at 230 nm (●), 343 nm (○) and conductivity (×). The first peak (elution volume 100 ml), which contained the thiol peptides was pooled and lyophilized. The peak with elution volume of about 150 ml is Tris buffer while the components with elution volumes of about 200 ml, 240 ml and 290 ml are believed to be mercaptoethanol, mercaptoethanol disulphide and thiopyridone, respectively.

Fractionation and analysis of thiol peptides. In a large-scale preparation, peptic thiol peptides were purified to homogeneity in a two-step procedure involving gel filtration on Sephadex G-25 and column electrophoresis as described elsewhere[8]. A preparation of tryptic thiol peptides, obtained in essentially the same way, was fractionated by Sephadex G-25 gel filtration followed by ion-exchange chromatography on an SP-Sephadex C-25 column in ammonium acetate at pH 5.0. The breakthrough peak in the chromatography was finally chromatographed on a QAE-Sephadex A-25 column at pH 8.5 with a linear gradient of ammonium acetate.

In the peptic digest each of the three gel filtration peaks were fractionated by electrophoresis. In this way a total of 12 peptides of 5 to 33 residues in length were obtained. Their composition and sequences[8], showed them to be derived from five different areas in the protein. Four of these were present in yields of 26–43 %. In the tryptic digest only the major gel filtration peak was further fractionated to give four major components. The composition of these are reported in Table I. When compared to the known amino acid sequences of the C-terminal half of ceruloplasmin[11,12], two of these (B-3 and B-10) agree with what is expected from tryptic splits around two of the cysteines, while tryptic peptides B-7 and B-16 can be accounted for as elongations of the previously sequenced peptic peptides A II and B II (ref. 8). The presently known sequences around these regions in ceruloplasmin are summarized in Fig. 6. It is clear that some of the enzymatic splits are only two residues away from the point of attachment. The gel matrix thus does not constitute a hindrance in the proteolytic digestion.

Isolation of disulphide peptides. One of the peptides (A III) in the peptic digest contained two cysteine residues. Thus it was possible that only one of these was

TABLE I

AMINO ACID COMPOSITION OF THE TRYPTIC THIOL PEPTIDES OBTAINED FROM HUMAN CERULOPLASMIN BY COVALENT CHROMATOGRAPHY

Values in parentheses are nearest integer (peptides B-7 and B-16) or composition of the expected tryptic peptides around the corresponding cysteinyl residues in the two C-terminal proteolytic fragments[11,12] (peptides B-3 and B-10). Values for carboxymethyl-cysteine and methionine were consistently too low.

Amino acid	*B-3*		*B-7*		*B-10*		*B-16*	
CM-Cysteine	0.70	(1)	0.72	(1)	0.26	(1)	0.72	(1)
Aspartic acid	3.03	(3)	4.72	(5)	4.87	(5)	5.32	(5)
Threonine	5.02	(5)	1.87	(2)	6.83	(8)	1.32	(1)
Serine			1.21	(1)	1.36	(1)	2.02	(2)
Glutamic acid	2.96	(3)	2.79	(3)	3.13	(3)	5.31	(5)
Proline	0.51	(0)	1.68	(2)	1.97	(2)	1.17	(1)
Glycine	2.96	(3)	1.32	(1)	3.15	(3)		
Alanine	0.96	(1)	2.37	(2)	1.21	(1)		
Valine	1.73	(2)	0.99	(1)	1.16	(1)	1.04	(1)
Methionine	0.63	(1)	0.69	(1)	1.94	(2)		
Isoleucine	1.12	(1)	1.32	(1)				
Leucine	2.67	(3)	3.79	(4)	3.77	(3)		
Tyrosine	0.84	(1)	0.66	(1)	0.88	(1)	0.95	(1)
Phenylalanine			1.47	(1)	1.78	(2)	1.04	(1)
Histidine	3.45	(4)	0.80	(1)	1.69	(2)		
Lysine	1.04	(1)	0.98	(1)	1.09	(1)	2.12	(2)
Tryptophan						(1)		
Arginine							0.80	(1)
Sum		29		28		37		21

```
Region 1    (FPZTYBTLZMVPBABGIA)LLSCQNLNHKAGL
            ↑                 ↑↑  |    ↑  ↑
            T                  P  SH   T  P

Region 2    DTANLFPQTSLTLHMWPDTTEGTFNVECLTTDHYTGGMKQKYTVNQCRRQSGDSTF
            ↑                       ↑  |         ↑        |        ↑
            T                       P  SH        T        S        P
                                                          |
                                                          S
                                                          |
                                               YGNQPGLTMCKGDS
                                               ↑            ↑
                                               P            P

Region 3    NIKTYCSEPEKVDKDNED(FZZSBR)
            ↑  ↑ |           ↑       ↑
            P  T SH          P       T

Region 4    LLHCHVTDHIHAGMETTYTVLQNEDTK
            ↑↑ |          ↑           ↑
            TP SH         P           T
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Fig. 6. *Suggested structure of the four regions in human ceruloplasmin that contain cysteine.* The one letter codon of amino acids is used. Arrows with a T or P indicate tryptic and peptic splits, respectively. The double arrows mark places of alternative peptic splits. The peptic peptides were sequenced as reported elsewhere[8]. The tryptic peptides, for which only compositions were obtained, have been written so as to conform to the known sequences of the peptic peptides and the corresponding parts of the two C-terminal proteolytic fragments of ceruloplasmin containing regions 2 and 4[11,12]. Stretches of unknown sequence, shown in parentheses, have been arranged so as to maximize internal homology[18]. Region 1 contains peptides B II and B-7, region 2 peptides A III and B-10, region 3 peptides A II and B-16 and region 4 peptides B I and B-3, where the Roman numerals refer to peptic peptides and Arabic numerals to tryptic peptides. The sequences in regions 1, 2 and 4 have been shown to be homologous[8].

attached originally to the matrix while the other was involved in a disulphide bridge. Since there is a tryptic split point between the two cysteines this possibility could be tested by a separate experiment. The peptic digest was prepared as above and the incubation mixture washed. Before reductive elution a second digestion was performed, this time with trypsin as the enzyme. From the supernatant a disulphide peptide was purified by Sephadex G-25 gel filtration[8]. The analysis of the peptide identified the peptic peptides which formed the disulphide (Fig. 6).

DISCUSSION

Covalent chromatography has proven to be a valuable addition to the arsenal of preparative procedures used in protein and peptide work. It has been used mainly in connection with protein purifications[13–15] while its use for preparation of thiol peptides has not been as common. The original work in this area[2] had several shortcomings, *e.g.*, the thiol gel contained as the spacer arm glutathione which was slowly released and contaminated the peptide preparations. Since the presently used gel has a hydroxypropyl spacer this problem has been remedied.

The originally rather poor coupling yields with ceruloplasmin (20–30%) have been increased to nearly quantitative reaction by an optimization of the reaction conditions. The avoidance of oxidation of thiol groups by the released cupric copper at pH 8 is of particular importance. The higher degree of substitution of the presently used thiopropyl gel has certainly also contributed.

The thiol peptides obtained are recovered together with a large amount of thiopyridone which complicates the transfer of cysteine to a stable derivative. Previously the mixture was oxidized to obtain cysteic acid. Since this reaction simultaneously destroys tryptophan, it is not ideal. The quantitative alkylation of the mixture was made difficult by the large amount of thiopyridone. A safe excess of alkylating agent is not desirable since histidine and methionine side chains might be alkylated. The final solution to the problem was to remove the reagents, prior to alkylation, by chromatography on Sephadex LH-20. This hydrophobic gel seems to exclude all charged peptides regardless of size while the neutral reagents were retarded. The possibility of selective elution of non-used activated structures on the thiopropyl gel by a low concentration of dithioerythritol[4] before elution of the peptides has not been used.

In its present form, covalent chromatography was proved to be an excellent and easy method for obtaining thiol peptides from ceruloplasmin. The potential of the method is illustrated by the results. Two different digests contained peptides that together allowed the sequences around the cysteinyl residues to be extended to 21–53 residues for the different peptides and account for a total of more than 150 residues from four regions. The use of new proteolytic enzymes probably would enlarge these areas even further. Several methionine residues were placed in sequence which is of value for ordering cyanogen bromide peptides. For proteins with relatively few disulphides it might be an equally useful tactic to use reduced protein for the preparation of thiol peptides, or reduce the peptides obtained from the non-reductive elution after the digestion for attachment to the activated gel.

The isolation of four different tryptic thiol peptides seems to contradict other analytical results which indicated that ceruloplasmin contains three cysteines[16] only

two of which are essential for copper-binding[17]. A possible explanation might be that an intermolecular thiol disulphide exchange allows the third cysteine to change identity.

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