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SEPARATION OF LIMITED TRYPTIC FRAGMENTS OF HUMAN CERULOPLASMIN BY GEL-PERMEATION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Limited tryptic proteolysis of human ceruloplasmin rapidly produces several large, protease-resistant fragments, suggesting that the molecule consists of several domains. In order to locate the sites of proteolytic cleavage in the whole molecule, we used gel-permeation high-performance liquid chromatography to determine the optimum conditions for fragment separation. Using a buffer containing 8 *M* urea, the 67,000-daltons tryptic fragment from single-chain ceruloplasmin was isolated in a sufficiently pure state for amino acid sequence analysis to determine its location in the uncleaved molecule. These results have been used in conjunction with amino acid sequence data to develop a schematic model of the domain structure of human ceruloplasmin.

INTRODUCTION

An important objective in the structural study of ceruloplasmin, a plasma glycoprotein [molecular weight *ca.* 135,000 daltons (135 kDal)] that can bind six or seven copper ions per molecule, is the analysis of the domain structure of the molecule. Ceruloplasmin is a single-chain protein[1–3], but if care is not taken to inhibit protease activity, it is often isolated in a proteolytically cleaved state consisting of three major fragments with molecular weights of 67, 50, and 19 kDal. These three fragments are arranged in the single-chain molecule with the 67-kDal piece at the N-terminus, the 19 kDal piece at the C-terminus, and the 50-kDal piece in between (Fig. 1)[2]. Amino acid sequence analysis has shown a three-fold internal homology for the entire molecule, with *ca.* 30% identity in sequence between the three homology units, each of *ca.* 340 residues[4,5]. Furthermore, limited proteolysis of the undenatured protein rapidly produces a series of discrete, relatively stable fragments, suggesting that the ceruloplasmin molecule consists of six (or possibly nine) domains[2,4]. Even after limited proteolysis, however, strong intramolecular interactions exist between the individual fragments, with retention of some but not all of the copper-binding ability[6]. In order to determine the domain structure of the protein, we have used gel-permeation high-performance liquid chromatography (HPLC) to separate the domain-size fragments produced by limited tryptic digestion of single-chain ceruloplasmin.

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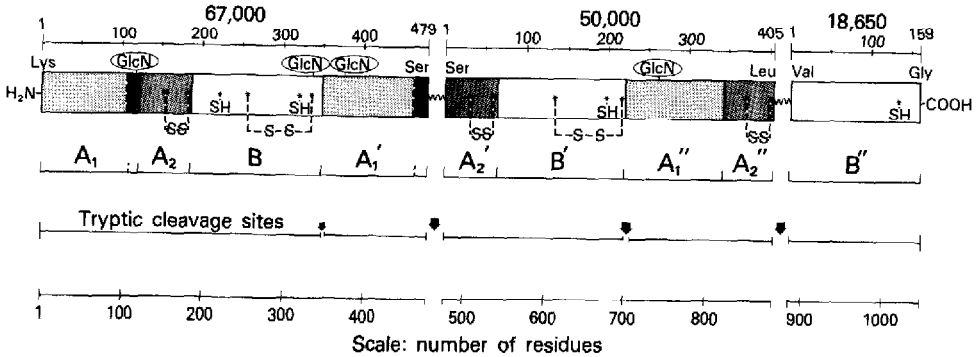

Fig. 1. Structural model of human ceruloplasmin (from ref. 4). The three fragments (molecular weights 67,000, 50,000 and 18,650) from the autolytically degraded sample are aligned in the proposed order. The shading indicates the proposed domain structure of the molecule, consisting of three sets of either two (A, B) or three (A_1, A_2, B) domains. The sites of tryptic cleavage in the single-chain molecule are indicated, the broad arrows indicating the three major sites, and the smaller arrow indicating the slower cleavage site in the 67 kDal fragment. Evidence for the disulfide bond in domain B' and for the four free cysteine sulfhydryls has been presented by Rydén and Norder[7]; the remaining disulfides have been predicted based on amino-acid sequence homology[4] and data presented here. The fragment labelled 18,650 is referred to as the 19-kDal fragment in the text.

EXPERIMENTAL

Protein

As described previously[2,6,8], the ceruloplasmin samples (Cp1 and Cp4) differed in the extent of proteolysis of the single-chain protein, and the A_{610}/A_{280} ratios of these samples were quite variable (ranging from 0.030 to 0.045). Therefore, in order to standardize the samples, they were initially chromatographed on a 55 × 50 mm I.D. column of iminodiacetic acid-activated Sepharose 4B (metal-chelate affinity chromatography) (Pharmacia, Uppsala, Sweden) that had been charged with Zn^{2+} ions[6].

Limited tryptic digestion

Trypsin, treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK), was from Worthington (Freehold, NJ, U.S.A.). The different ceruloplasmin samples, both the intact, single-chain (Cp4) and the fragmented (Cp1) forms, were digested at 37°C with a 1:100 enzyme–substrate ratio. Digestion was stopped by addition of soybean trypsin inhibitor (Worthington) in a 2:1 inhibitor–enzyme ratio. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with a 5% stacking gel and a 12–30% acrylamide gradient separation gel in a Tris–glycine, pH 8.0, system, was used to examine the extent of digestion (modified procedure of Laemmli[9]). The protein bands were stained with Coomassie Blue, and the electropherograms were analyzed by densitometry, using a Helena Quick-Scan Densitometer.

Separation of limited tryptic fragments

Ceruloplasmin samples investigated included (1) the autolytically fragmented sample (Cp1), and (2) a 10-min limited tryptic digest of single-chain ceruloplasmin

(Cp4). The different samples were chromatographed on an Altex Spherogel TSK-G2000SW or G3000SW gel-permeation column (600 × 7.5 mm I.D.), using a Beckman pump, Model 110A, and a Gilson Holochrome UV monitor connected to a Linear Recorder, Model 261/MM. Several eluents were investigated: 0.1% trifluoroacetic acid (TFA) (Pierce, Rockford, IL, U.S.A.; Sequanal Grade), pH 2.1; 0.1% TFA–10% 1-propanol (Burdick & Jackson, Muskegon, MI, U.S.A.; Chromatography Grade), pH 2.1; 0.1% TFA–0.1% Triton X-100 (Sigma, St. Louis, MO, U.S.A.), pH 2.1; 0.05 *M* sodium acetate–0.2 *M* sodium sulfate, pH 5.5; 0.1 *M* Tris · HCl–0.15 *M* sodium chloride, pH 8.0; 0.1 *M* Tris · HCl–0.1% SDS, pH 8.0; 8 *M* urea–0.1 *M* Tris · HCl–0.15 *M* sodium chloride, pH 8.0. Manual Edman sequence analysis was performed on the purified fragments using the method of van Eerd and Takahashi[10], and the phenylthiohydantoin-amino acids were identified by HPLC.

RESULTS AND DISCUSSION

Standardization of ceruloplasmin samples

In order to minimize the number of minor cleavage products from tryptic digestion, we removed the apoceruloplasmin from our samples, since it is more extensively cleaved during proteolysis than copper-containing holoceruloplasmin[11]. All ceruloplasmin samples were therefore standardized by preliminary chromatography on a Zn^{2+}-equilibrated metal-chelate affinity column (Fig. 2). Material eluted from the column by the starting buffer (pH 8.0) was identified as ceruloplasmin by SDS-PAGE and immunodiffusion analysis. This fraction was not used in any subsequent experiments, however, because it did not show the absorbance at 610 nm which is due to blue Type 1 Cu^{2+}. Blue copper-containing ceruloplasmin was eluted from the column at pH 6.0, and all fractions with an A_{610}/A_{280} ratio greater than 0.041 were pooled. This material, representing 60–80% of the initial protein, was used in all further experiments.

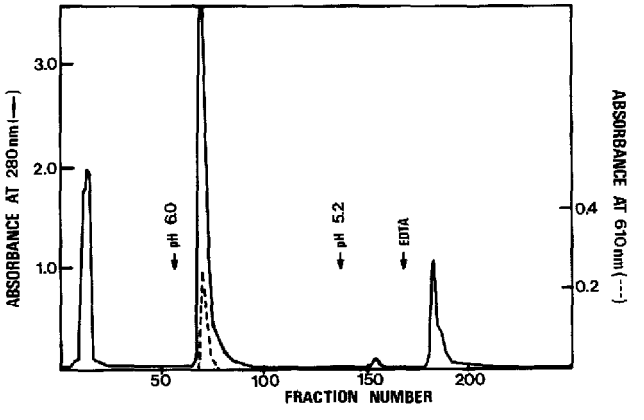

Fig. 2. Standardization of ceruloplasmin samples. Autolytically fragmented Cp1 (130 mg) was applied to a Zn^{2+}-equilibrated metal-chelate affinity column in 0.1 *M* Tris · HCl–0.15 *M* sodium chloride, pH 8.0. The column was then washed with the following buffers in a stepwise manner: 0.1 *M* sodium phosphate–0.15 *M* sodium chloride, pH 6.0; 0.1 *M* sodium acetate–0.15 *M* sodium chloride, pH 5.2; and 0.05 *M* EDTA, which stripped the column of metal ions and any remaining protein. The chromatogram is essentially the same for single-chain ceruloplasmin (Cp4).

Limited tryptic digestion

Limited tryptic proteolysis of undenatured single-chain ceruloplasmin rapidly produces several fragments that are resistant to further digestion (Fig. 3A)[6,12]. After 10 min of tryptic digestion, there were four major fragments produced, with molecular weights of 67, 26, 25, and 19 kDal (*cf.* Fig. 1). As proteolysis continued, the 67-kDal fragment was also cleaved, producing a 49-kDal fragment, and several smaller fragments of *ca.* 20 kDal and lower began to appear. We have also found that limited tryptic proteolysis of the autolytically degraded sample produces several protease-resistant fragments, and that these fragments have molecular weights similar to those produced from the single-chain protein (Fig. 3B)[6]. Quantitative analysis by densitometry of the SDS-PAGE patterns resulting from limited tryptic digestion of Cp1 and Cp4 has allowed us to localize the tryptic cleavage sites in the protein (Fig. 1)[2,4], but determination of the actual cleavage sites in the single-chain molecule requires separation and identification of the individual fragments.

Fragment separation

Separation of large, domain-size fragments of human ceruloplasmin has generally been hindered by strong intramolecular interactions that tend to hold the fragments together[6]. Although there are no disulfide bonds between the three major fragments in Cp1[2], previous separations of these fragments for sequence analysis employed reduction and alkylation of the cysteine residues in addition to strong denaturing reagents (*e.g.*, urea or guanidine · HCl)[13]. In addition to identifying the individual fragments produced by limited tryptic proteolysis of the single-chain molecule, we are also interested in determining the disulfide bonds within each fragment.

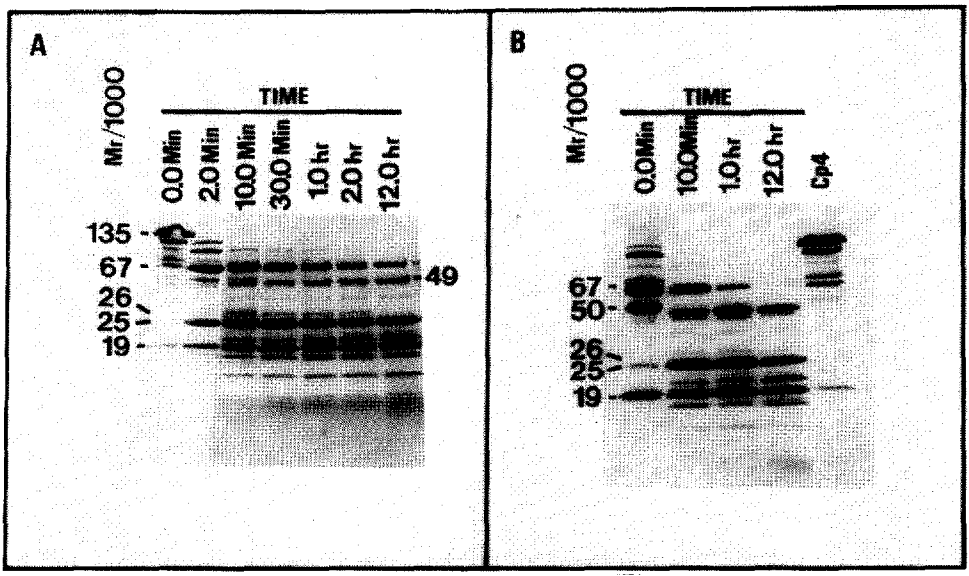

Fig. 3. Limited tryptic digestion of (A) single-chain ceruloplasmin (Cp4) and (B) the spontaneously cleaved preparation (Cp1). In (B), the 50-kDal fragment has been completely cleaved to 26- and 25-kDal fragments by 10 min, and a small amount of the 49-kDal fragment, resulting from cleavage of the 67-kDal fragment, is present. The last lane in (B) contains Cp4, for comparison between (A) and (B). The fragments are identified by Mr/1000 (Mr = molecular weight).

Therefore, we investigated the use of gel-permeation HPLC to determine the optimum conditions for separation of the tryptic fragments without modification of the disulfide bonds or free sulfhydryl groups. This approach proved most advantageous, because we were able to investigate rapidly several different eluents for their ability to separate the fragments using a minimal amount of sample for each experiment.

Of the various eluents investigated, the only buffer with which we could fractionate any of the samples was 8 *M* urea–0.1 *M* Tris · HCl–0.15 *M* sodium chloride, pH 8.0. The autolytically degraded sample (Cp1) was partially resolved, but a considerable amount of the sample was aggregated (Figs. 4a and 5a). The fragments generated in a 10-min tryptic digestion of single-chain ceruloplasmin did not aggregate, however, and the 67-kDal fragment was clearly separated from the smaller pieces on a TSK-G3000SW column (Figs. 4b and 5b). Densitometric analysis of the electropherogram (Fig. 5b) indicated that the 67-kDal fragment was 90% pure, and amino acid analysis of this fragment matched analysis results obtained for the 67-kDal fragment from Cp1 (ref. 14). Furthermore, the amino-terminal sequence of this fragment was Lys-Glu-Lys-His-Tyr-Tyr, as determined by manual Edman degradation, which matches the amino-terminal sequence of the single-chain protein[15]. These results clearly locate the 67-kDal fragment resulting from limited tryptic digestion of whole ceruloplasmin in the amino-terminal half of the protein, similar to the 67-kDal fragment present in the autolytically cleaved sample.

Previously, only two of the limited tryptic fragments had been identified by sequence analysis: (1) Rydén[12,16] identified a cleavage site in the 67-kDal fragment

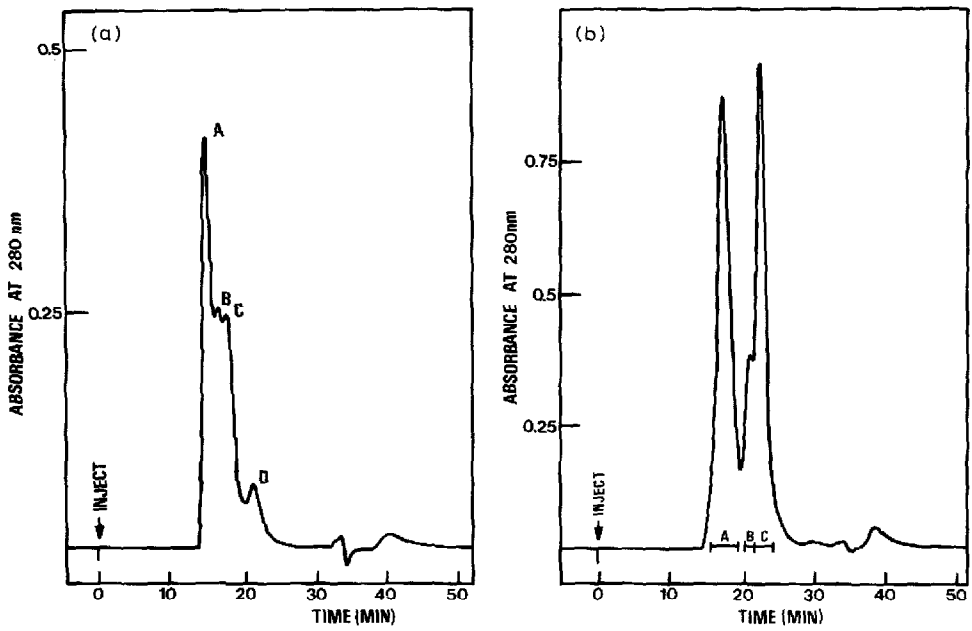

Fig. 4. Gel-permeation HPLC of ceruloplasmin fragments. A TSK-G3000SW column in 8 *M* urea–0.1 *M* Tris · HCl–0.15 *M* sodium chloride, pH 8.0, was used to fractionate (a) the autolytically degraded sample (Cp1), and (b) a 10-min limited tryptic digestion of Cp4. The flow-rate was 1 ml/min, and the sample (1 mg) was injected in 250 μl of the column buffer.

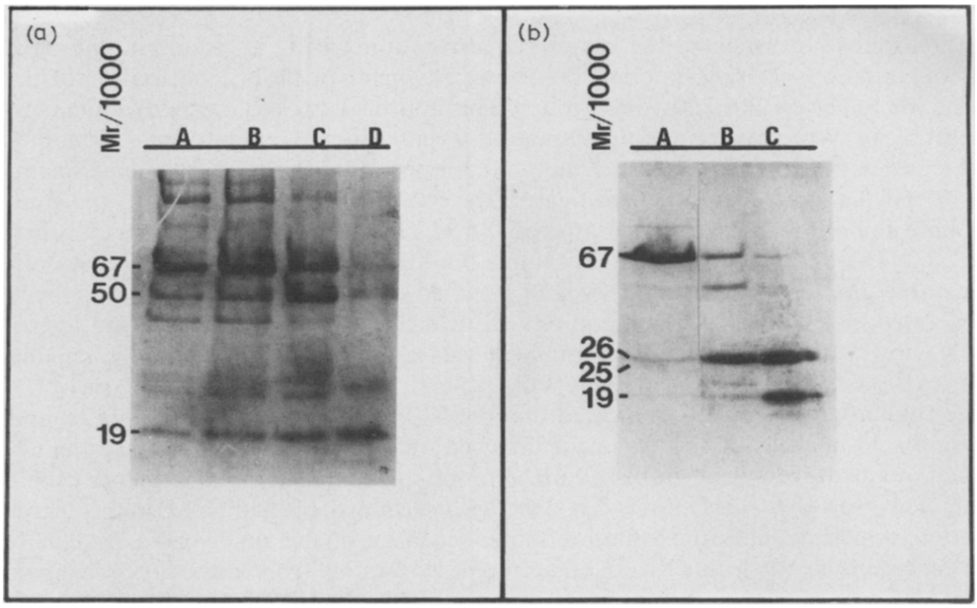

Fig. 5. SDS-PAGE analysis of Figs. 4a (a) and 4b (b). Lower-case letters correspond to the chromatograms in Fig. 4; capital letters indicate the corresponding peaks.

that occurred near residue 340 of the whole molecule, and (2) Prozorovski *et al.*[17] identified a 19-kDal tryptic fragment that matched the fragment of the same molecular weight observed in Cp1. These results alone could not definitively align the fragments, however, without identification of one of the larger tryptic fragments. Consequently, our identification of the 67-kDal tryptic fragment has enabled us to determine the order of the fragments resulting from limited proteolysis of single-chain ceruloplasmin. These results were used in the development of the schematic model of the domain structure of the molecule that had been predicted by amino acid sequence data[4,5].

CONCLUSION

Trypsin rapidly cleaves undenatured, single-chain ceruloplasmin into several protease-resistant fragments, suggesting that the molecule consists of several domains. Comparison of the tryptic fragments of single-chain ceruloplasmin with the tryptic fragments of autolytically degraded ceruloplasmin enabled us to predict the interdomain junctions in the whole molecule, but accurate determination of the cleavage sites requires separation and identification of the fragments. Separation of large fragments from ceruloplasmin is often hindered by strong intramolecular interactions, however, which tend to cause fragment aggregation. Gel-permeation HPLC is a technique whereby several eluents could be rapidly investigated with minimal amounts of sample in order to determine the best conditions for fragment separation. By using gel-permeation HPLC with 8 *M* urea, the 67-kDal tryptic fragment was sufficiently purified for identification by amino acid sequence analysis. These results have been used in conjunction with amino acid sequence data to develop a structural model of the ceruloplasmin molecule.

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