A C T A U N I V E R S I T A T I S L O D Z I E N S I S
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PRODUCTS OF TRYPTIC PROTEOLYSIS OF HUMAN AND PORCINE CERULOPLASMINS

Obtained by us tryptic digestion products of human and porcine ceruloplasmins were fractionated on a Sephadex G-50 column. The mixture of peptides was separated into four fractions (peaks) which were characterized for molecular weight as well as content of protein, copper, sulfhydryl groups and disulfide bridges.

Results show quite serious differences in elutions profiles of peptides obtained after tryptic digestion of human and porcine ceruloplasmins suggesting that porcine one is more sensitive to proteolytic attack than human. Relatively low molecular weights for obtained peptides in case of both human and porcine ceruloplasmins after long-lasting trypsin hydrolysis show that both are very sensitive to proteolysis what one must take into account during isolation procedure.

Analysis of copper content in peptide fractions showed that some of them were very enriched in copper and other contained small or only trace amounts. Thus several atoms of copper (probably four) must be located close to each other in native Cp molecule what can be connected with the fact that they take part in the reaction catalyzed by ceruloplasmin.

Ceruloplasmin (Cp, ferroxidase, iron (II): oxygen oxidoreductase, EC 1.16.3.1) is a blue multifunctional copper-containing enzyme present in vertebrate plasma. It is a single chain glycoprotein of a molecular weight of about 130 000 which is composed of six domains of two different kinds or possibly nine do-

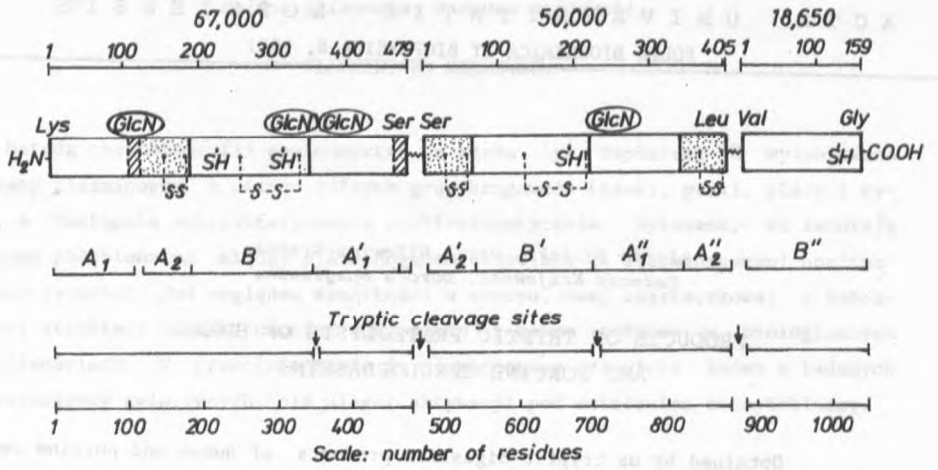

Fig. 1. Structural model of human ceruloplasmin molecule. In three fragments, i.e. 67 000 (479 amino acid residues), 50 000 (405 residues) and 18 650 (159 residues) 4 sulfhydryl groups, 5 disulfide bridges, N-terminal and C-terminal amino acids and glucosamines of carbohydrate chains (GlcN) are designed. Proposed series of two (A, B) or three (A_1, A_2, B) sets repeating homologous domains are distinguished by prime marks: A', A'', A''' etc. (according to T a k a h a s h i et al. [26])

mains of three kinds (Fig. 1). Ceruloplasmin contains 0.28-0.32% copper by weight [8, 22, 28] which corresponds to 6-7 copper atoms per molecule present in three spectroscopically (EPR) distinguishable types of binding sites (types 1, 2 and 3). The nature of specific ligand environments of copper is the subject of intense investigations [2, 4-6, 13, 25]. The location of six copper binding sites is still obscure. Moreover, so far no data concerning location of copper atoms in other kind of Cp molecule.

The present study was undertaken to examine the tryptic proteolysis products of human and porcine Cp molecules and elucidate the location of copper atoms in Cp.

MATERIALS AND METHODS

The commercial human Cp preparation were purchased from Vaccine and Serum Laboratory in Warsaw and further purified in our Laboratory by gel filtration chromatography on a Sephadex G-200

column. The porcine Cp used for our experimental study was isolated from fresh blood obtained from a slaughterhouse and collected in the presence of both 0.02M sodium citrate and 6-aminocaproic acid. Plasma was separated by centrifugation and used directly for the preparation of enzyme by means of the elaborated in our Department method. This procedure allowed to obtain undegraded Cp preparation with high degree of chemical purity. The purity was tested by absorbance ratio A_{610}/A_{280}, contents of bound copper and polyacralamide gel electrophoresis.

PREPARATION OF PORCINE CERULOPLASMIN

Step 1. Adsorption on DEAE-Sephadex A-50

Porcine plasma was 4-fold diluted with water and then applied to a DEAE-Sephadex A-50 column (4 cm x 20-25 cm) equilibrated with 50 mM K-phosphate buffer, pH 6.8. Flow rate 300-400 ml/h, temp. 5°C. Cp was adsorbed at the top of the column as a strong blue colored band. The gel in the column was then washed with 80 mM K-phosphate buffer, pH 6.8, containing 20 mM 6-aminocaproic acid, until the A_{280} was below 0.1 in the eluate. Cp was then eluted with 0.3 M acetate buffer, pH 5.5-5.6, containing 0.2 M NaCl at flow rate 50-100 ml/h. The blue colored fractions were collected.

Step 2. Precipitation with ammonium sulfate

To the blue colored Cp solution 0.4 volumes of saturated ammonium sulfate was added dropwise with stirring (to ~ 30% saturation). The mixture was cooled at 5°C for 15 min. and then centrifuged (4000 g, 15 min.). The pellet was discarded. To the supernatant a volume of saturated ammonium sulfate equal to the initial Cp solution volume was added, achieving a final ammonium sulfate addition 1.4 times the initial volume of Cp solution (58% saturation). The mixture was cooled at 5°C for 30 min. and then centrifuged (4000 g, 15 min.). The pellet was collected and dissolved in 100 ml (per 1 l of plasma) of 0.2 M acetate buffer, pH 5.6, containing 0.125 M NaCl. After dissolving the precipitate we calculate the volume of the precipitate by subtracting the initial volume of acetate buffer from the final volume of

the solution. Then enough acetate buffer was added to obtain a final volume corresponding to ten times the volume of the precipitate.

Step 3. Precipitation with an ethanol-chloroform mixture

To the Cp containing solution 3 volumes of ethanol-chloroform (9:1, v/v) mixture were added dropwise with gentle stirring for 60 min. period at room temperature. Then the mixture was set aside for 100 min. at room temperature and the precipitate was collected by centrifugation (4000 g, 15 min.). The Cp was extracted from the sediment with 10-20 ml of 0.15 M NaCl, pH 6.8--7.0, at constant stirring for 5 min. and the extract was cleared by centrifugation at 16 000 g, 60 min. Dialysis against 0.15 M NaCl pH 6.8-7.0 was used to remove traces of ethanol and chloroform. Non-ceruloplasmin copper contaminations were removed on a column of Chelex-100 resin and the Cp preparation was stored at 5°C.

ANALYTICAL PROCEDURES

Tryptic digestion of Cp was carried out with 100 mg of protein and 4 mg of trypsin in 5 ml of 0.2 M ammonium bicarbonate, at pH 8.7 for 20 h at 37°C (18). The products were lyophilized, dissolved in 1 ml of 0.05 M ammonium acetate buffer, pH 7.0 and fractionated on a Sephadex G-50 column (1 x 50 cm) equilibrated with the same buffer. Peptides eluted from the column (fractions 2 ml/tube) were detected by absorbance at 280 nm. The copper content of each fraction was estimated by a colorimetric method as described below. Copper-rich fractions were pooled and characterized for molecular weight as well as content of protein, copper, -SH groups and disulfide bridges.

Both protein and peptide concentrations were determined by the biuret method as described by L a y n e [12] with bovine serum albumine as a standard. Ceruloplasmin concentrations were also evaluated spectrophotometrically at 280 nm or 610 nm using an experimentally obtained absorption coefficient of $A^{1\%}_{280} = 14.35$ (human Cp) and 13.8 (porcine Cp) or $A^{1\%}_{610} = 0.66$ and 0.75, respectively.

Copper content determinations were performed according to a modified method of G u b l e r et al. [7] with sodium diethyldithiocarbamate in cuvettes with 5 cm path lenght which allowed a five fold higher sensitivity of the method.

Polyacrylamide gel electrophoresis (PAGE) was conducted in 7% gel at pH 8.3 under non-denaturing conditions by the procedure of M a r c e a u and A s p i n [14]. Proteins in the gel were stained with 0.04% Coomassie brilliant blue G-250 in 3.5% $HClO_4$ [19]. To detect the Cp oxidase activity, gels were incubated with o-dianisidine reagent [16].

The cysteine residues (free -SH groups) in Cp molecule and peptides were determined with Ellman reagent (DTNB, 5,5´-dithiobis (2-nitrobenzoic acid) as follows: to 4.9 ml of protein solution (3-5 mg) in 0.1 M phosphate buffer, pH 8.0, was added 0.1 ml of 10 mM DTNB solution in the same buffer and after 2 min. absorbance value at 412 nm was measured against blank. Cysteine hydrochloride and reduced glutathion were used as a standards. The content of disulfide groups was determined by means the method of A n d o and S t e i n e r [1] with Ellman reagent.

The molecular weight (M_r) for each copper rich peptide fraction was determined by gel filtration on a Sephadex G-50 column (1 x 50 cm) equilibrated with 0.05 M ammonium acetate buffer, pH 7.0. The dextran blue (M_r > 40 000), cytochrom c (M_r = 12 600) and potassium chromate (M_r = 194) were used as a molecular weight standards.

RESULTS

Undegraded human and porcine Cp preparations obtained by our method possessed a high degree of chemical purity. It was shown by absorbance ratio A_{610}/A_{280} and copper content (see Table 1). Further confirmation was obtained in polyacrylamide gel electrophoresis (the presence of bands which not only were stained with Coomassie briliant blue but exhibited ceruloplasmin oxidase activity when incubated with o-dianisidine reagent as well).

Table 1

Some biochemical parameters of ceruloplasmin preparations

Parameter \ Preparation	Ceruloplasmin			
	human		porcine	
	value		value	
	theoretical	obtained (n=3)	theoretical	obtained (n=4)
A_{610}/A_{280}	0.046	0.0452* ±0.002	0.054	0.0528* ±0.0012
µg Cu/mg Cp	2.80	2.76 ±0.01	3.20	3.14 ±0.016

* Mean value of n preparations ± standard deviation.

Table 2

Contents of copper, sulfhydryl and disulfide groups in peptide fractions obtaining after tryptic digestion of human ceruloplasmin

Fraction	Molecular weight	Mol Cu/ /mol Cp*	Mol-SH/ /mol Cp*	Mol-S-S/ /mol Cp*
I	> 40 000	8.11 ±1.32**	0.56 ±0.11	4.04 ±0.72
II	10 000	3.50 ±0.65	1.13 ±0.13	0.05 ±0.03
III	4 220	1.28 ±0.36	1.06 ±0.09	2.01 ±0.37
IV	708	0.94 ±0.23	2.20 ±0.16	1.43 ±0.26

* The values in peptides were recalculated for mol Cp.

** The values in Table correspond to mean values of 3 experiments ± standard deviations.

Three human and four porcine Cp preparations were used for tryptic digestion under experimental conditions described above. Tryptic digestion products of Cp were completely soluble in 0.05 M ammonium acetate buffer, pH 7.0.

Gel filtration chromatography of these products from human Cp on Sephadex G-50 is shown in Fig. 2. The peptide absorbance pattern at 280 nm includes a peak at the void volume due to

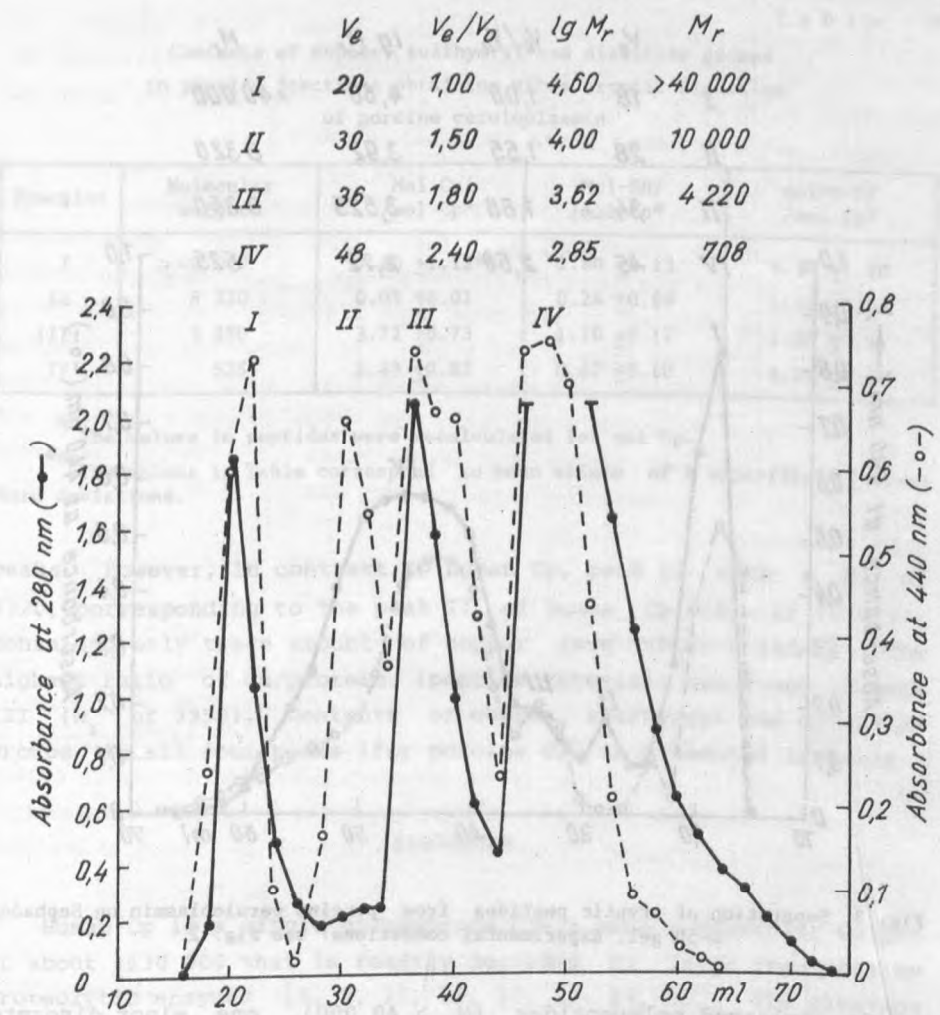

Fig. 2. Separation of tryptic peptides from human ceruloplasmin on Sephadex G-50. Column (1 x 50 cm) equilibrated with 50 mM ammonium acetate, pH 7.0 and eluted with the same buffer. Flow rate 15 ml/h. Fractions (2 ml/tube) were collected and monitored by absorbance at 280 nm for peptide elution pattern and by reaction with sodium diethyldithiocarbamate (A_{440nm}) for copper elution pattern determination

	V_e	V_e/V_o	lg M_r	M_r
I	18	1,00	4,60	>40 000
II	28	1,55	3,92	8 320
III	34	1,88	3,525	3 350
IV	45	2,50	2,72	525

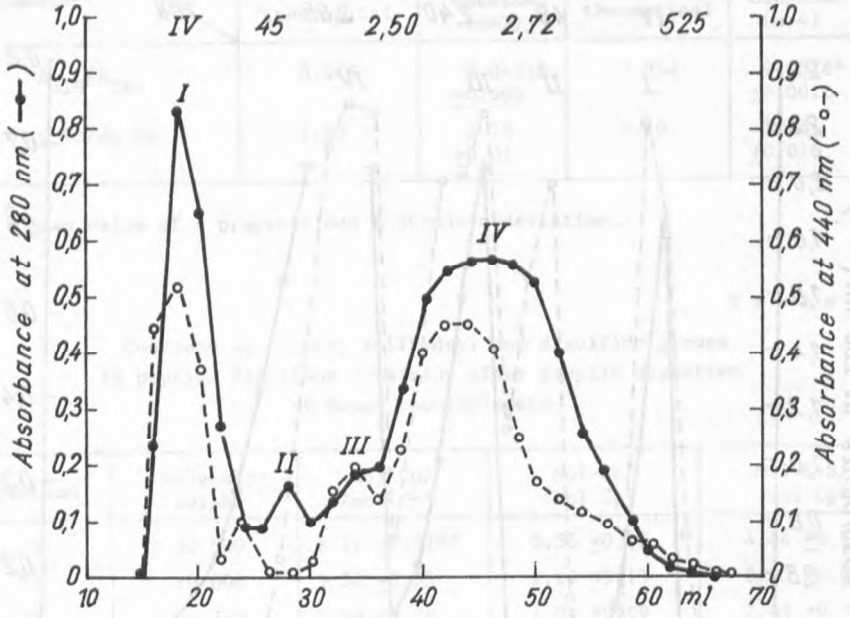

Fig. 3. Separation of tryptic peptides from porcine ceruloplasmin on Sephadex G-50 gel. Experimental conditions: see Fig. 2

mainly uncleaved polypeptides (M_r > 40 000), one minor discrete peak (M_r of 10 000) and two well-separated peaks which followed (M_r of 4200 and 708). Copper determinations showed the first minor discrete peak (peak II) was highly enriched in copper and followed by other copper peaks. Contents of copper, sulfhydryl and disulfide groups in received four peaks is presented in Table 2.

Gel filtration chromatography of tryptic proteolysis products from porcine Cp on Sephadex G-50 is shown in Fig. 3. Alike human Cp we can observe a peak at the void volume and three other

Table 3

Contents of copper, sulfhydryl and disulfide groups in peptide fractions obtaining after tryptic digestion of porcine ceruloplasmin

Fraction	Molecular weight	Mol Cu/ /mol Cp*	Mol-SH/ /mol Cp*	mol-S-S/ /mol Cp*
I	>40 000	7.80 ±1.12**	1.80 ±0.13	6.30 ±1.30
II	8 320	0.05 ±0.01	0.24 ±0.09	1.40 ±0.05
III	3 350	3.72 ±0.73	1.10 ±0.17	1.20 ±0.31
IV	525	2.43 ±0.62	0.62 ±0.10	4.20 ±0.43

* The values in peptides were recalculated for mol Cp.

** The values in Table correspond to mean values of 8 experiments ± standard deviations.

peaks. However, in contrast to human Cp, peak II with a M_r of 8320, corresponding to the peak II of human Cp (M_r of 10 000), contained only trace amount of copper (see Tables 2 and 3). The highest ratio of Cu/protein (peptide material) was found in peak III (M_r of 3350). Contents of copper, sulfhydryl and disulfide groups in all four peaks (for porcine Cp) is presented in Table 3.

DISCUSSION

Human Cp is a single polypeptide chain with a molecular weight of about 130 000 that is readily degraded to large fragments by proteolytic enzymes [3, 9, 11, 15, 17, 20, 24, 26]. The cleavage of the Cp molecule appears to be a specific process. There are a few major sites in the molecule that are exposed and susceptible to the proteolytic enzymes. Limited enzymatic cleavage of human Cp to yield 67 000, 50 000 and 19 000 daltons fragments occurs at specific exposed sites at random structure in between domain-like regions [3, 10, 26]. Numerous observations indicate that the human Cp molecule may be composed of a six of domain-like structures of two different kinds A and B or possibly nine domains of three kinds A_1, A_2 and B (Fig. 1) [26].

In Cp molecule there are 6-7 copper atoms which appear to exist in three (or possibly four) different ligand environments, i.e. type 1, type 2 and type 3 [2, 4, 5, 6, 13, 25]. The location of these copper binding sites is still obscure.

According to R y d e n [21] four of six copper atoms are localized in 19-kDal fragment from the carboxyl terminus of the human Cp molecule (see Fig. 1). These are copper atoms which take part in the reaction catalyzed by Cp. They form the active site which is made up of one blue type 1 copper (so called 1a or fast), one non-blue type 2 and a pair of magnetically coupled type 3 coppers. These are situated in such a way that electrons can be accepted from the substrates by type 1 and 2 coppers and transferred to the copper pair which binds the oxygen molecule and reduces it. Other two copper atoms are in 50-kDal and 67-kDal fragments (see Fig. 1).

Above mentioned R y d e n's results have not got any confirmation. R a j u [18] obtained two stable copper-containing peptide fractions with an apparent M_r of 11 kDal and 1 kDal, as result of 18 h tryptic digestion of human Cp. The first fragment derived from the 19-kDal fragment of the Cp molecule (see Fig. 1) was found to contain 50% of the Cu atoms of the Cp molecule, i.e. 3 Cu atoms per molecule. Other three copper atoms were detected in the 1-kDal peptides fraction and probably they would be located in 67-kDal fragment of Cp molecule designed on our Fig. 1 as B.

Above presented discrepancies between results obtained by R y d e n and R a j u encouraged us to carry out comparative studies on both human and porcine ceruloplasmin preparations.

Comparative analysis of our results (Fig. 2) with R a j u data (Fig. 4) indicates that there are some similarities in respect to elution pattern. Namely we obtained a peak (peak II) with M_r of 10 kDal (corresponding to 11 kDal obtained by R a j u) highly enriched in copper. However, in contrast to this author, who obtained only one peak containing peptides of M_r 1 kDal, we received two low molecular weight well-separated peaks, both containing copper. Found by us data concerning contents of copper in peak of M_r 10 kDal (3.5 mol Cu/mol Cp) would be rather more close to the results presented by R y d e n [21],

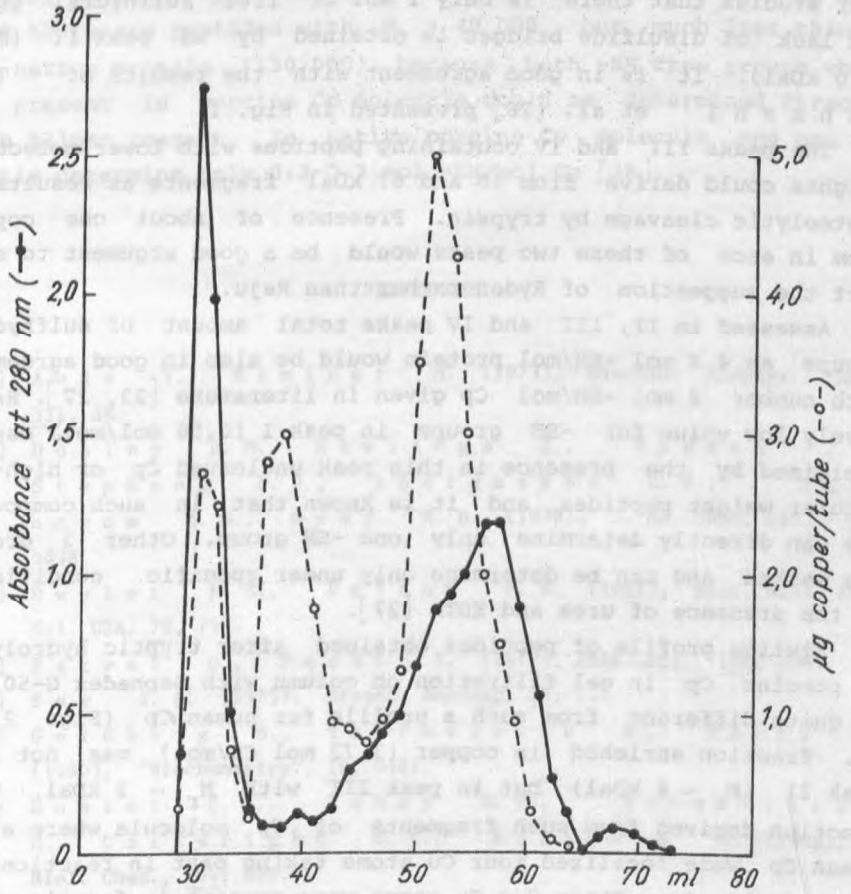

Fig. 4. Separation of tryptic peptides from human ceruloplasmin on Sephadex G-50. Column (1.5 x 50 cm) equilibrated with 50 mM ammonium acetate, pH 7.0 and eluted with the same buffer. Flow rate 10 ml/h. Fractions (1.5 ml/tube) were collected and monitored by absorbance at 280 nm for peptide elution pattern and by reaction with bathocuproinedisulfonic acid for copper elution pattern determination (according to R a j u [18])

who estabilished the presence of four Cu atoms in 19-kDal fragment of Cp molecule (see Fig. 1). This fact could support an idea that our fraction of M_r 10-kDal also was derived from this 19--kDal fragment. This supposition was also confirmed by our fur-

ther studies that there is only 1 mol of free sulfhydryl group and lack of disulfide bridges in obtained by us peak II (M_r = = 10 kDal). It is in good agreement with the results of T a-k a h a s h i et al. [26] presented in Fig. 1.

The peaks III and IV containing peptides with lower molecular weights could derive from 50 and 67 kDal fragments as results of proteolytic cleavage by trypsin. Presence of about one copper atom in each of these two peaks would be a good argument to support the suggestion of Ryden rather than Raju.

Assessed in II, III and IV peaks total amount of sulfhydryl groups as 4.4 mol -SH/mol protein would be also in good agreement with number 4 mol -SH/mol Cp given in literature [23, 27]. Relatively low volue for -SH groups in peak I (0.56 mol/mol) can be explained by the presence in this peak uncleaved Cp or high-molecular weight peptides and it is known that in such compounds one can directly determine only one -SH group. Other 3 groups are masked and can be determine only under specific conditions, in the presence of urea and EDTA [27].

Elution profile of peptides obtained after tryptic hydrolysis of porcine Cp in gel filtration on column with Sephadex G-50 gel is quite different from such a profile for human Cp (Figs 2 and 3). Fraction enriched in copper (3.72 mol Cu/mol) was not in peak II ($M_r \sim 8$ kDal) but in peak III with $M_r \sim 3$ kDal. This fraction derived from such fragments of Cp molecule where alike human Cp were localized four Cu atoms taking part in reaction catalyzed by Cp. Other 2-3 Cu atoms were present in fraction with very low M_r (~ 500).

Above mentioned results show that location of Cu atoms in both kinds of Cp (human and porcine) is rather similar. So far we do not know a molecular structure of porcine Cp in detail (in contrast to human Cp) and therefore it is difficult to state strictly in which fragment of molecule these Cu atoms exist.

Comparison of elution profiles for peptides obtained after tryptic digestion of human and porcine Cp presented in Figs 2 and 3 (very large peak IV in comparison with peaks II and III in case of porcine Cp) shows that porcine is more sensitive to proteolytic attack and it has to possess more bounds very susceptible to trypsin. Observed by us amount 2 moles -SH groups in peak I in

case of porcine Cp confirms this as well. It means that in this peak there are peptides with M_r > 40 000, but much less than M_r for native protein (130 000), because both -SH free groups which are present in porcine Cp molecule could be determined directly with Ellman reagent. In native porcine Cp molecule one can directly determine only 0.2-0.3 mol -SH/mol Cp [28].

REFERENCES

[1] Ando Y., Steiner M. (1973), Biochim. Biophys. Acta, 311, 38.

[2] Dooley D. M., Rawlings I., Dawson J. H., Stephens P. J., Andreasson L. E., Malmstrom B. G., Gray H. B. (1979), J. Am. Chem. Soc., 101, 5038.

[3] Dwulet F. E., Putnam F. W. (1981), Proc. Natl. Acad. Sci. USA, 78, 790.

[4] Farver O., Pecht I. (1979), FEBS Lett., 108, 436.

[5] Fee J. A. (1975), Struct. Bonding. 23, 1.

[6] Goldberg M., Vuk-Pavlovic S., Pecht I. (1980), "Biochemistry", 19, 5181.

[7] Gubler C. J., Lahey M. E., Ashenbrucker H., Cartwright G. E., Wintrobe M. (1952), J. Biol. Chem., 196, 209.

[8] Kasper C. B., Deutsch H. F. (1963), J. Biol. Chem., 238, 2325.

[9] Kingston I. B., Kingston B. L., Putnam F. W. (1977), Prac. Natl. Acad. Sci. USA, 74, 5377.

[10] Kingston I. B., Kingston B. L., Putnam F. W. (1979), Proc. Natl. Acad. Sci. USA, 76, 1668.

[11] Kingston I. B., Kingston B. L., Putnam F. W. (1980), J. Biol. Chem., 255, 2878.

[12] Layne E. (1957), "Methods in Enzymology" (Colowick S. P., Kaplan N. O., Eds.) 3, 450, New York.

[13] Malkin R., Malmstrom B. G. (1970), Adv. Enzymol., 33, 177.

[14] Marceau N., Aspin N. (1973), Biochim. Biophys. Acta, 328, 351.

[15] Moshkov K. A., Lakatos S., Hajdu J., Zavodsky P., Neifakh S. A. (1979), Eur. J. Biochem., 94, 127.

[16] Owen J. A., Smith H. (1961), Clin. Chim. Acta, 6, 441.

[17] Prozorowski V. N., Rashkovetski L. G., Shavlovski M. M., Vasiliev V. B., Neifakh S. A. (1982), Int. J. Peptide Protein Res., 19, 40.

[18] Raju K. S. (1983), Mol. Cell. Biochem., 56, 81.

[19] Reisner A. H., Nemes P., Bucholtz C., (1975), Anal. Biochem., 64, 509.

[20] Ryden L., (1872), Eur. J. Biochem., 26, 380.

[21] Ryden L., (1982), Proc. Natl. Acad. Sci. USA, 79, 6767.

[22] Ryden L., Bjork I., (1976), "Biochemistry", 15, 3411.

[23] Ryden L., Norder H., (1981), J. Chromatogr., 215, 341.

[24] Samsonidze T. G., Moshkov K. A., Kiselov N. A., Neifakh S. A., (1979), Int. J. Protein Res., 14, 161.

[25] Siiman O., Young N. M., Carey P. R., (1976), J. Am. Chem. Soc., 98, 744.

[26] Takahashi N., Bauman R. A., Ortel T. L., Dwulet F. E., Wang C-C, Putnam F. W. (1983), Proc. Natl. Acad. Sci. USA, 80, 115.

[27] Witwicki J., Zakrzewski K. (1969), Eur. J. Biochem., 10, 284.

[28] Zgirski A. (1986), *Ceruloplazmina człowieka, świni i krowy. Stabilność i alternatywna droga działania*, Łódź.

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PRODUKTY TRYPSYNOWEJ PROTEOLIZY CERULOPLAZMINY CZŁOWIEKA I ŚWINI

Otrzymane produkty trypsynowego rozpadu ceruloplazminy człowieka i świni frakcjonowano za pomocą filtracji żelowej na kolumnie z żelem Sephadex G-50. Mieszanina peptydów rozdzielała się na 4 frakcje, które były analizowane pod względem masy cząsteczkowej, zawartości miedzi, grup -SH i mostków disiarczkowych. Wykazano dość znaczne różnice w profilu elucyjnym mieszaniny peptydów otrzymanych z ceruloplazminy człowieka i świni, świadczące o znacznie większej podatności tej ostatniej na działanie trypsyny. Względnie niskie masy cząsteczkowe dla peptydów otrzymanych po długo trwającej hydrolizie trypsynowej zarówno ceruloplazminy człowieka, jak i świni wskazują, że cząsteczki te są bardzo wrażliwe na proteolizę, co powinno być brane pod uwagę podczas procedury izolowania.

Analiza zawartości miedzi w poszczególnych frakcjach wykazała, że niektóre z nich zawierały bardzo dużo miedzi, a inne ilości małe bądź śladowe. Świadczy to, że w natywnej cząsteczce kilka atomów miedzi (prawdopodobnie cztery) biorące udział w procesie katalitycznym znajdują się bardzo blisko siebie.