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The Components of Orosomucoid

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Orosomucoid has been prepared by fractional precipitation with perchloric acid followed by chromatography on DEAE-cellulose. All the components of which the protein is built up, have been determined. Special attention is paid to the amino acids and amide nitrogen. The number of carbohydrate and amino acid residues in orosomucoid are calculated.

Extensive studies have been made on the chemistry of orosomucoid. This protein is interesting because of its high carbohydrate content (40 %), and because the structure, and especially the linkage between the protein and the carbohydrate is unknown. The individual carbohydrate components of orosomucoid are well known; however, fewer data have been published about the amino acid part.

Amino acid analysis of orosomucoid presents special problems. During the acid hydrolysis of this protein a massive humin formation makes the results uncertain. It is shown that humin formation does not take place in the presence of titanium trichloride.

EXPERIMENTAL

Preparation of orosomucoid

The preparation of seromucoids from human serum by fractional precipitation with perchloric acid and fractionation on DEAE-cellulose has been described previously [1]. Three fractions were obtained, called A, B and C. Fraction B was eluted with 0.05 M phosphate + 0.1 N sodium chloride, pH 6.8, and consisted of two components in the ratio of about 9:1, called B-1 and B-2. When fraction B was rechromatographed in the same system three times, the B-1 fraction became pure as tested by paper electrophoresis and immunologically by the method of double diffusion in agar according to Ouchterlony [2,3]. Fraction B-1 was shown to be identical with orosomucoid tested immunologically and by determination of hexose, hexosamine and nitrogen [1].

An almost ash-free preparation was obtained by passing the protein through a column of Dowex 2 in OH-form followed by Dowex 50 in H-form. The preparation was finally lyophilized.

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Table 1.

Hydrolysis	Weighted protein-N mg	Analyzed N in the hydrolysate mg	Per cent recovery	Mean
24 h 110°C	17.0	15.8	93	95
44 h 110°C	19.5	18.9	95	
72 h 110°C	18.0	17.3	96	
24 h, 110°C with $TiCl_3$	15.0	15.2	101	99
44 h, 110°C with $TiCl_3$	18.4	17.3	94	
72 h, 110°C with $TiCl_3$	17.8	18.2	102	

Amino acid analysis

The amino acids were determined according to Moore, Spackman and Stein [4] by chromatography on Amberlite IR-120. Cysteine plus cystine was determined by means of hydrazinolysis [5] and tryptophane with *p*-dimethylaminobenzaldehyde and sodium nitrite as described by Spies and Chambers [6].

Special attention was paid to the hydrolysis of the protein because of the humin formed. It was observed that all the sialic acid and a large part of the hexosamine was destroyed. It could also be expected that part of the amino acids had been lost. An attempt was made, therefore, to reduce the humin formation by means of catalysts. Titaniumtrichloride, of the metal salts tried, was found to eliminate the humin without destruction of amino acids.

The hydrolysis was performed as follows: In six Pyrex test tubes about 15 mg of orosomucoid with a nitrogen content of 10.7 % was dissolved in 2 ml redistilled 6 N hydrochloric acid. To three of the tubes were added three drops of titaniumtrichloride ($TiCl_3$, 15 %, Merck). The tubes were evacuated and sealed, and hydrolysis was carried out at 110 ± 2°C for 25, 44, and 72 h. The tubes without $TiCl_3$ contained a considerable amount of humin, which seemed to be coal particles. In the tubes with $TiCl_3$, however, the solutions were almost clear, and the small amount of particles were of inorganic origin.

The contents were transferred quantitatively through filter papers into small beakers by means of distilled water and dried in a desiccator over sodium hydroxide. Finally the residues were dissolved in 5 ml of pH 3.25 buffer for chromatography. Aliquots were taken out for nitrogen determination (micro Kjeldahl). The results are given in Table 1. The difference from the theoretical values was about 5 % and may be due to technical errors during the different procedures. There is, however, a tendency to better yield when $TiCl_3$ is used. This also applies to the amino acid analysis.

Chromatography was performed with 1 ml hydrolysate on the long column and 0.6 or 0.7 ml on the short one. In Table 2 the values are given as amino acid residues in per cent of hydrolyzed protein. Glucosamine which emerged as a free peak from the short column before lysine, had undergone partial destruction and was found in amounts of about 5 %. Ammonia was found in amounts of 13—15 %, given as ammonia nitrogen in per cent of hydrolyzed nitrogen. These values are not given in the table.

Table. 2

Hydrolysis time and conditions	24 h	24 h +$TiCl_3$	44 h	44 h +$TiCl_3$	72 h	72 h +$TiCl_3$	Extrapolated values of residues	Moles per 46 000 g orosomucoid	Grams per 100 g protein
Components	% amino acid residue of orosomucoid								
Aspartic acid	6.40	6.56	6.08	6.69	6.11	6.11	6.5	26	7.5
Threonine	4.31	4.31	3.91	4.52	4.02	3.99	4.3	19	4.7
Serine	1.71	1.66	1.37	1.69	1.57	1.55	1.7	9	2.1
Glutamic acid	8.95	8.55	8.30	8.92	8.39	8.88	8.9	31	10.1
Proline	2.45	2.48	2.10	2.63	2.35	2.47	2.5	12	3.0
Glycine	1.16	1.25	1.13	1.25	1.08	1.08	1.2	9	1.6
Alanine	2.33	2.30	1.81	1.96	1.86	1.88	2.3	15	2.8
Valine	2.13	2.18	2.28	2.38	2.22	2.46	2.3	11	2.7
Methionine	0.42	0.44			0.44	0.79	0.5	2	0.5
Isoleucine	2.64	2.73	2.73	2.86	2.69	2.70	2.7	11	3.1
Leucine	4.50	4.74	(3.33)	4.85	4.72	4.74	4.7	19	5.4
Tyrosine	3.70	3.92	3.43	3.79	3.84	3.59	3.8	11	4.3
Phenylalanine	3.96	4.05	(3.14)	3.94	3.69	(3.24)	4.0	12	4.5
Lysine	4.71	4.53	4.52	4.80	4.98	(3.43)	4.7	17	5.5
Histidine	1.07	1.29	1.12	0.97	1.24	0.87	1.2	4	1.4
Arginine	2.84	2.94	3.19	3.38	3.17	2.80	3.1	9	3.5
Tryptophan							1.4	4	1.5
Cystine/2							1.4	6	1.7
Acetyl glucosamine							12.8	29	14.0
N-acetyl neuraminic acid							12.0	19	12.8
Hexoses							14.6	41	16.2
Fucose							1.0	3	1.1

The amino acids have also been determined microbiologically by Weimer *et al.*[7] and by Caputo and Santoro [8] by means of high voltage electrophoresis. The agreement between these values and those in Table 2 is not very good.

Determination of amide nitrogen

The amide nitrogen content of orosomucoid is extremely difficult to determine due to the instability of sialic acid during hydrolysis, and because amino sugars as well as sialic acid are deaminated to some degree under the alkaline conditions generally used for liberation of ammonia.

Five different procedures for hydrolysis have been used in this study.

1. *Hydrolysis in 1 N sulphuric acid for 30 and 60 min. in sealed tubes at 120°C.* The hydrolysates were transferred to Conway chambers and made alkaline by addition of sodium hydroxide with phenolphthalein as indicator. The liberated ammonia was determined by titration after diffusion into boric acid for 5 h at 4°C. At this low temperature there is no destruction of hexosamine under alkaline conditions (Tracey [9]). This procedure gave a massive humin formation. The values found were equivalent to 13 to 20 residues per 46 000 g of protein.

2. *Hydrolysis in 0.1 N sulphuric acid for 1 to 4 h at 80°C.* This procedure has been used by Zilliken *et al.*[10] for liberation of sialic acid. Liberated ammonia was determined by diffusion in Conway chambers as described under procedure 1.

After half an hour of hydrolysis the liberated ammonia was less than 1 residue. After 2 and 4 h of hydrolysis the values rose to about 2 residues. Humin formation could be seen after about 2 h.

3. *Hydrolysis according to Rees* [11] *with 10 N hydrochloric acid for different time periods at 37°C, followed by diffusion in Conway chambers.* The humin formation started after some hours. After 4 days of hydrolysis, about 5 residues were liberated, after 8 days about 10 residues, and then the values rose slowly to about 22 residues after 28 days.

4. *Hydrolysis is 10 N hydrochloric acid as under procedure 3, but with addition of* $TiCl_3$. The humin formation was inhibited and first seen after 12 days. The amount of ammonia liberated was greater the first days compared with procedure 3, after 25 days it was about the same. After 2, 5, 12, and 25 days 4, 12, 14, and 19 moles of ammonia were liberated per 46 000 g of orosomucoid.

5. *Hydrolysis in 2 N HCl at 110°C.* Humin was formed after one hour and 19 residues were found. The values then rose slowly to 23 residues after 3 h and were found nearly constant for the following 3 h.

Thus it has not been possible to hydrolyze orosomucoid without destruction of sialic acid, and the liberated ammonia is more an expression of destruction than of amide nitrogen. According to Popenoe and Drew [12] a modified orosomucoid may be obtained by splitting off the sialic acid by means of sialidase from *Clostridium perfringens*. This modified protein has a slightly lower molecular weight, but as far as we know there is no other chemical change in the molecule than the absence of sialic acid. The modified protein was prepared as described by Popenoe and Drew [12]. Sialic acid was split off to a degree of 85 %, and the reaction mixture was separated on DEAE-Cephadex * in phosphate buffer pH 6.2. By stepwise elution with increasing ionic strength a fraction was obtained that was homogeneous in paper electrophoresis and free of sialic acid. This protein was used for determination of amide nitrogen as described under procedures 3 and 5. Only traces of humin were formed.

With procedure 3 hydrolysis was performed up to 10 days. After one day of hydrolysis 3—4 residues were liberated. After 2 days the number was 7, and this value was almost constant up to 6 days. After 10 days, however, the liberated ammonia corresponded to about 12 residues per 40 500 g of modified orosomucoid. The value 40 500 is calculated from the assumed molecular weight of orosomucoid by substraction of 19 sialic acid residues.

With procedure 5, *i.e.* 2 N HCl and 110°C, about 10 residues were found after one hour of hydrolysis. The value rose slowly to about 12 residues after 3—6 h. In all experiments the values after more than one hour of hydrolysis were within 12 ± 2 residues or 0.42 ± 0.07 per cent amide nitrogen.

With the assumption that the only change in orosomucoid treated with sialidase, is that sialic acid has been split off, it may be concluded that the number of 12 ± 2 amide groups are also present in native orosomucoid.

Determination of acid groups

This was determined by the dye-binding method of Fraenkel—Conrat and Cooper [13]. Using safranine 0 at pH 11.5 a total number of 83 ± 2 acid groups was found.

Basic groups could not be determined as orosomucoid was not precipitated by the dye orange G at pH 2.2.

Determinations of the carbohydrate components

Hexosamine was determined by the method of Morgan and Elson as described by Svennerholm [14]. Hexoses by anthrone [15], sialic acid by Bials reaction according to Svennerholm [16] and fucose by the cysteine method of Gibbons [17]. The hexosamines were found to be only glucosamine, determined by means of chromatography on Dowex 50 according to Gardell [18]. Test for glucoronic acid was negative. The values are given in Table 3 together with those of some other authors.

* DEAE-Cephadex was a gift from Pharmacia, Uppsala, Sweden.

DISCUSSION

All the components of which orosomucoid is built up, are now known. The number of residues are given in Table 3, assuming a molecular weight of 46 000. This value agrees well with the values calculated from the minimum molecular weight for each component. It is also known that glucosamine as well as sialic acid is N-acetylated. Sialic acid can be split off by enzymes from *Clostridium perfringens* [12] or other micro-organisms without affecting the rest of the molecule. About the same was obtained by cold concentrated hydrochloric acid [24]. It seems likely, therefore, that sialic acid is in end-position.

No N terminal amino acid could be found by the fluorodinitrobenzene- or the phenyl*iso*cyanate-method [25].

The C-terminal amino acid was found by Schmid *et al.*[24] to be serine. Only one mole C-terminal amino acid was found, indicating a single peptide chain.

The most interesting problem is the number of carbohydrate groups and the nature of linkage. One of the possibilities is the linkage to amino groups. Popenoe and Drew [12] found, however, 17 amino nitrogen groups by the method af Van Slyke [26], and the same value was found by the author. Thus the free amino groups correspond to the 17 ε-amino groups of lysine. Still the possibility exists that the carbohydrate component is linked to the N-terminal amino group as a single polysaccharide chain. This is not very likely, however.

Another possibility is linkage to one of the acid groups. Linkage between the phenol group in tyrosine and carbohydrate or between SH-groups and carbohydrate, has not been reported, but linkage between dicarboxylic amino acids and carbohydrate, has been found in egg albumin by Johansen *et al.*[27]. They were able to isolate a fragment containing only carbohydrate and aspartic acid.

The number of acid groups in orosomucoid are 26 β-aspartic acid carboxyl groups, 31 β-glutamic acid carboxyl groups, 19 sialic acids, 11 phenol groups

Table 3.

Author	Nitrogen %	Hexosamine %	Hexose %	N-acetyl neuraminic acid %	Fucose %	Mol. weight
Weimer *et al.*[7]	10.1	11.9	16.4			
Smith *et al.*[19]						44 100
Schmid [20]	10.7	11.5	17.2			
Werner and Odin [21]		11.5	14.1	9.8		
Schultze [22]		11.3[a]	14.7	12.1	0.7	41 000
Winzler [23]	10.1	11.9	16.4	10.6	1.3	44 100
Caputo and Santoro [8]	11.1	10	17			41 000[b]
						46 000[c]
Present author	10.7	11.3	16.2	12.6	1.1	

a) Recalculated from 13.9 % N-acetyl hexosamine.
b) By ultracentrifugation.
c) By refractive index measurements.

of tyrosine, 6 SH-groups of cysteine and 1 carboxyl group of the C-terminal serine. 12 of the β-carboxyl groups are bound to amide groups, and the calculated total number of free acid groups are thus about 82. The dye-binding method gave a value of 83 ± 2 free acid groups, which is in agreement with the value of 86 ± 2 found by Popenoe and Drew [12]. Thus, there is no real difference between calculated and estimated acid groups. The value of the acid groups is not exact, as it has been calculated from many different determinations, all of which are connected with errors. If the assumed linkage between carbohydrate and β-carboxyl exists, however, the number of polysaccharide units is very low.

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