

Preparation of Crystalline Cytochrome c from Beef Heart Muscle

SVEN PALÉUS

Medicinska Nobelinstitutet, Biokemiska avdelningen, Karolinska Institutet, Stockholm, Sweden

A simple method for the crystallization of beef heart cytochrome c is described. Amino acid analysis together with a number of physico-chemical characteristics of the preparation are reported. An explanation for previous discrepancies in the sulphur content of cytochrome c is given.

For some years we have been concerned with the development of a simple, reproducible method¹ of preparing crystalline mammalian cytochrome c*. As early as 1950 we introduced a new chromatographic procedure² in the preparation of cytochrome c. This involved the use of the ion-exchange resin Amberlite IRC-50 and much of the procedure to be described is based on the use of this carboxylic acid type resin. Japanese biochemists³⁻⁶ who have recently carried out a great deal of work with cytochrome c from a variety of sources have taken advantage of this ion exchange treatment during the purification and concentration procedure. The acid extraction of cytochrome c was introduced by Theorell⁷ and was also employed in the classical modifications by Keilin and Hartree^{8,9}. The latter workers, however, used trichloroacetic acid both for the extraction and the final precipitation of the pigment. There has been some question, however, as to the effect of TCA on cyt.c and as we¹⁰ had already found H₂SO₄ to give a better yield in the extraction of salmon heart cyt.c this acid was accordingly used for the extraction of beef heart cyt.c.

EXPERIMENTAL

Assay method. The concentration of cyt.c was determined spectrophotometrically after reduction with sodium dithionite in 0.065 M phosphate buffer, pH 6.8 using $E_1^{1\text{ cm}} = 550\text{ m}\mu = 22.7$, corresponding to cytochrome c of 0.435 % iron content.

* The following abbreviations will be used: cyt.c = cytochrome c, TCA = trichloroacetic acid.

Preparation of resin

(A) *Amberlite*. 1/2 kg Amberlite XE-64 was suspended in 1.12 N NaOH and allowed to stand in a water-bath (50°) for 6 h with occasional stirring. The supernatant was discarded and the resin washed with distilled water until neutral. 5 l of 5 % H₂SO₄ were then added and the mixture after stirring allowed to stand for 1/2 h. The resin was next washed with distilled water until neutral and then suspended in 5 l of 5 % ammonium hydroxide and allowed to stand for 1 h with occasional stirring. Finally the resin was washed with redistilled water until neutral and filtered on a Büchner funnel until nearly dry (20 % moisture content). The resin was stored in this state.

(B) *Duolite*. 10 g of Duolite CS-101 was ground by hand using a pestle and mortar and suspended in 200 ml 2.5 % H₂SO₄. It was then washed with distilled water until neutral; at the same time the finest particles were removed by decantation. The ammonium salt of the resin was formed by standing for 1/2 h with occasional stirring in 250 ml of 5 % ammonium hydroxide. It was then washed with distilled water until neutral. The regeneration procedure was repeated once, using redistilled water instead of distilled water as formerly. It was filtered and stored damp as for the Amberlite.

Preparation and purification of cytochrome c

Step 1. 6 kg fresh cow hearts were minced, 15 l of cold distilled water added and the pH adjusted to 4.1 with 1 N H₂SO₄. This and the subsequent steps were carried out at 4°. The mixture was allowed to stand for about 1 h with occasional stirring. After filtration through cheese cloth the mince was extracted further with 6 l of water. The total extract was brought to pH 7 with 4 N ammonia, allowed to stand for 1 1/2 h, then centrifuged (2 000 g) and the supernatant filtered through Whatman No. 1 paper. This resulted in a volume of 20.7 l, which contained 132 g total protein determined by the biuret reaction as modified by Gornall *et al.*¹¹

Step 2. 198 g of Amberlite XE-64 were added and the mixture stirred occasionally over 2 h. The resin which by then had taken up the cyt.c was washed with about 50 l distilled water on a Büchner funnel. The cyt.c. was eluted in a total volume of 3 400 ml 0.15 M phosphate buffer, pH 7. It was then dialyzed against 65 l of 0.002 M ammonium hydroxide and centrifuged. The final volume was 3 425 ml with a total protein content of 6.2 g and a cyt.c. content of 925 mg as determined by spectrophotometric assay.

Step 3. The preparation was next concentrated by adsorption on 10 g of Amberlite XE-64. The resin was washed with about 400 ml 0.002 M ammonium hydroxide and the cyt.c. eluted in 475 ml 0.09 M phosphate buffer, pH 7.5.

Step 4. Am₂SO₄ was now added in a concentration of 0.6 g/ml, the preparation allowed to stand overnight and filtered through a Whatman No. 1 filter paper (14 cm diam.). The volume at this stage was 600 ml. The preparation was dialyzed against approx. 50 l 0.002 M ammonium hydroxide, and finally against 18 l of 0.05 M ammonium phosphate buffer, pH 7.

Step 5. The preparation (volume 850 ml) was next concentrated on a column (2 × 20 cm) of Duolite CS-101 and washed with 150 ml 0.05 M ammonium phosphate buffer pH 7 and eluted in 28 ml 0.4 M ammonium phosphate buffer pH 7. The total yield of cyt.c. was 436 mg.

Crystallization

346 mg of the above material was dialyzed against a total of 6 l of 0.002 M ammonium hydroxide in 3 changes. Part of this preparation was used for an iron content determination and other analyses; the remainder was dialyzed against 50 % saturated Am₂SO₄, pH 7, and finally against 98 % saturated Am₂SO₄, pH 7. The starting volume for crystallization was 12.5 ml and the concentration of cyt.c. was 23.9 mg/ml. 200 μl ammonium hydroxide (25 %) and 10 mg ascorbic acid were added; the pH as measured with the glass electrode was 8.5. After 12 h at room temperature there was a precipitate of rod-like crystals, which after several days were transformed into ball-like aggregates which have earlier been described by Hagihara *et al.*³ The crystals which were collected by centrifuga-

tion were in the reduced state. The supernatant (10 ml) was also completely reduced, and contained 4.26 mg cyt. c/ml. The yield of crystals was therefore 86 %. The crystals were dissolved in 0.8 ml distilled water, 5 mg ascorbic acid and, 150 μ l ammonium hydroxide (25 %) were added, and Am_2SO_4 was then added in solid form to 93 % saturation. Similar crystals were again obtained on standing; in this case the supernatant contained 20 mg cyt. c and the crystal yield here was 92 %. For a third crystallization the crystals were again collected by centrifugation. They were then washed with 5 ml 98 % saturated Am_2SO_4 -solution (pH 8) and dissolved to 5 ml with distilled water. 250 μ l ammonium hydroxide (25 %) and 5 mg ascorbic acid were added (pH 9.5) and then solid Am_2SO_4 to 80 % saturation whereupon precipitation occurred. Crystals (Fig. 1) formed after 1 1/2 days. There was again a tendency of the crystals, especially at first, to come together in ball-like aggregates. On standing, a partial transformation of the crystals into plates occurred as was described by Hagihara *et al.*⁴ The crystals were collected by centrifugation (87 % yield), dissolved in the minimum volume of water and dialyzed against distilled water until sulphate free.

Analytical results obtained on thrice crystallized cytochrome c

The iron content as determined by the modified Lorber method¹² was 0.43 %.

The homogeneity of the pigment was tested by electrophoresis in the Spinco model H instrument. The preparation was dissolved in phosphate buffer pH 7.08, ionic strength 0.1, and completely oxidized by careful addition of $\text{K}_3\text{Fe}(\text{CN})_6$. Only one symmetrical peak was obtained after 4 h running.

The extinction ratios $\frac{E_{550} \text{ m}\mu \text{ red}}{E_{280} \text{ m}\mu \text{ ox}} = 1.20$, $\frac{E_{550} \text{ m}\mu \text{ ox}}{E_{590} \text{ m}\mu \text{ red}} = 5.85$ and $E_1^1 \text{ cm } 550 \text{ m}\mu = 22.6$ were obtained.

The nitrogen content (Dumas) was 14.83 %. The sulphur content of the preparation at this stage was determined to be 1.42 %. However, after passage through a column (10 \times 2 cm) of Lewatite MIH (a weakly basic anion exchange resin from Farbenfabriken

Table 1. Amino acid composition of crystalline beef cytochrome c (0.43 % Fe; Mol. wt = 12 900; 14.83 % N — Dumas method). 2.5–5.5 mg of cyt.c hydrolyzed 20 and 70 h at 110 °C with 0.5 ml 5.7 N HCl in a sealed, evacuated tube. For a small (< 1 %) residue of black humin substance, correction was made in the 70 h sample.

Amino acid residue	Number per molecule			Amino acid residue	Number per molecule		
	Time of hydrolysis		According to Ehrenberg and Theorell ¹⁶		Time of hydrolysis		According to Ehrenberg and Theorell ¹⁶
	20 h	70 h		20 h	70 h		
Aspartic acid	7.93	8.11	9.4	Lysine	17.11	18.48	18.3
Serine	8.38	7.02	8.5	Histidine	2.83	3.20	3.1
Threonine				Arginine	1.98	2.05	3.4
Glutamic acid	11.62	11.9	11.9	Phenylalanine	4.17	4.06	3.3
Glycine	15.13	14.85	15.5	Methionine	1.68	1.01	
α -Alanine	6.04	6.07	6.6	Cysteine	2.0 *		
Valine	3.06	2.80	3.3	Tryptophane	1.0 **		
Proline	4.29	3.83	3.9				
Isoleucine	5.86	5.88	6.2				
Leucine	5.88	6.38	6.2				
Tyrosine	3.63	3.40	3.8				

* According to Paléus²¹

** According to Theorell and Åkesson¹⁸

Table 2. Summary of purification procedure of cytochrome c.

	Fe content %	Amount of cyt.c.*				Yield %	Purity $E_{1\text{cm}}^{1\%}$ 550 m μ
		Volume (ml)	mg/ml	total mg			
1. Extraction		20 700	—	—	—	—	
2. After concentration on XE-64, 1st time	0.17	3 425	0.27	925	100	6.7	
3. Concentration on XE-64, 2nd time		475	1.41	670	72	—	
4. After Am ₂ SO ₄ treatment	0.38	614	0.99	612	66	19.8	
5. After concentration and chro- matography on Duolite CS-101	0.41	28	15.57	436	47	21.8	
After third crystallization	0.43			284	31	22.6	

* Starting weight of minced beef heart — 6 kg.

Bayer, Leverkusen) this value was reduced to 1.05 %. An amino acid analysis according to Moore *et al.*¹³ is given in Table 1. A portion of the sample dissolved in 0.065 M phosphate buffer, pH 6.8, and reduced with a small excess of ascorbic acid in a loosely capped cuvette was still in the fully reduced state over a period of three days.

DISCUSSION

As already reported¹ the early method of preparing crystalline beef heart cyt. c by Hagihara *et al.*³ did not give a homogeneous preparation. The homogeneity of the preparation described here was indicated by the electrophoretical result and the yield of crystals calculated on the weight of the starting material has been nearly doubled over that obtained by the above workers. In Table 2 a summary has been given of the purification method. It should be noted how well the iron-content increases up to the value of 0.43 % in the last step and also how the $E_{1\text{cm}}^{1\%}$ 550 m μ -values conform with the iron-values. It was very difficult to obtain the high ratios of $\frac{E_{550\text{ m}\mu\text{ red}}}{E_{280\text{ m}\mu\text{ ox}}}$ as reported by Hagihara *et al.*³ The values were variable without apparent reason within the range 1.15—1.30. On the other hand the value of 5.85 for the ratio $\frac{E_{590\text{ m}\mu\text{ ox}}}{E_{590\text{ m}\mu\text{ red}}}$ was close to but slightly higher than the values reported by Henderson and Rawlinson¹⁴ and also Margoliash *et al.*¹⁵ for resin-purified but not crystallized material.

The amino acid analysis (Table 1) revealed values which agreed well in most cases with the results of Ehrenberg and Theorell¹⁶. There is a difference of one amino acid residue in the case of aspartic acid, arginine and phenylalanine; the difference in the case of glycine, serine and threonine is insignificant. Compared with the results of Takahashi *et al.*¹⁷ there are some further small differences, especially in the case of glutamic acid, serine and threonine, tyrosine and α -alanine. On the other hand the phenylalanine content is the

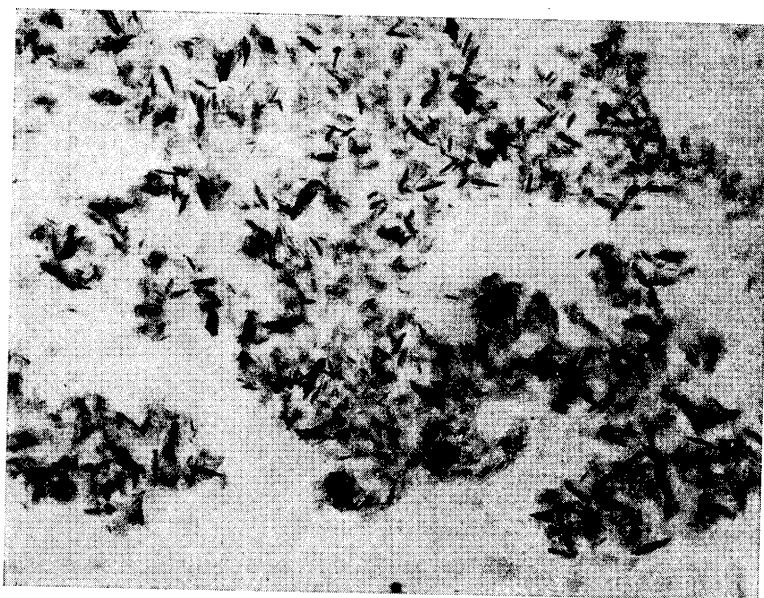


Fig. 1. Crystalline beef cytochrome c ($\times 430$), Crystal size, $2 \times 10 \mu$. Reduced form.

same in both preparations. It should be noted that the three above-mentioned analyses were carried out on samples of cyt.c prepared by different methods.

The nitrogen content (14.83 %) of the crystals was rather low and corresponded to 137 N-atoms per iron atom. This result was in conformity with other analyses carried out by the author on different preparations of cyt.c¹⁰. On adding the nitrogen-atoms of all the amino acids present (using the highest value from the 20 and 70 h hydrolyses) together with the four porphyrin-nitrogen atoms, 139 nitrogen-atoms (15.08 % N) are obtained. Earlier, Theorell and Åkeson¹⁸ had found 143 and Paul¹⁹ 145 nitrogen atoms per iron atom, but their preparations had been obtained by different methods.

The iron content of 0.43 % corresponds to a minimal molecular weight of 12 900, which value was used in the calculation of the amino acid content of the preparation.

Åkeson²⁰ showed that two methionine residues were present in cyt. c. Confirmation of this was obtained by the author²¹ but at the same time four sulphur atoms only were found to be present. The remaining two S-atoms were accounted for as the two cysteine residues shown by Theorell²² to be concerned in the prosthetic group linkage. There still remained, however, the discrepancy between the 5—6 sulphur atoms observed by Theorell and Åkeson¹⁸ and Åkeson²⁰. This appears now to be explained by the finding that the sulphur content is reduced from 6 to 4 atoms per cyt.c molecule by passage through an anionic resin. As no other significant change in the cytochrome c molecule has been observed by this treatment and as the author's former value

of 4 sulphur atoms per molecule was obtained after exhaustive dialysis it would seem likely that two salt linked sulphate ions are removed by the anionic resin.

Addendum: The sedimentation constant of this thrice crystallized material was $S_{20}^{\circ} = 1.62 \times 10^{-13} s$, very close to the value found in this laboratory for previous preparations²³. Determination of the molecular weight of crystalline beef cyt.c by Ehrenberg²⁴ has given a value of 13 300, which is in agreement with the value obtained above from the iron content (12 900).

Acknowledgements. The author wants to express his sincere gratitude to Professor H. Theorell and Dr. R. W. Henderson for kind criticism in connection with the work.

The work was supported by grants from *Statens naturvetenskapliga forskningsråd* and *Magn. Bergvalls Stiftelse*. It is a pleasure to thank Misses A.-M. Fridén and G. Tietgen for technical assistance.

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Received July 19, 1960.