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Cytochrome *c* Peroxidase

3. The Amino Acid Composition of Cytochrome *c* Peroxidase of Baker's Yeast

NILS ELLFOLK

Department of Biochemistry, University of Helsinki, Helsinki 17, Finland

The amino acid composition of crystalline cytochrome *c* peroxidase from baker's yeast has been determined with the aid of the amino acid analyzer (Beckman/Spinco 120B). Separate determinations have been made for tryptophan and amide ammonia. The analyses indicate the presence of the following amino acid residues: Asp_{42}, Thr_{14}, Ser_{14}, Glu_{27}, Pro_{15}, Gly_{23}, Ala_{16}, Val_{12}, Met_6, Ile_8, Leu_{23}, Tyr_{12}, Phe_{16}, Lys_{31}, His_5, Arg_9, Cys_1, Trp_6, $(-CONH_2)_{29}$. The molecular weight of 34 164 for cytochrome *c* peroxidase computed from the amino acid composition agrees well with the value previously obtained from physical measurements. A value of 0.727 ml/g for the partial specific volume was calculated from the weight percentages of the amino acid residues and their respective specific volumes. The electrophoretic behaviour of cytochrome *c* peroxidase can be satisfactorily explained on the basis of the amino acid composition. Cytochrome *c* peroxidase from baker's yeast differs distinctively from plant peroxidases in showing a high content of lysine, tyrosine, and tryptophan, whereas the cysteine content is very low.

The amino acid content of peroxidases has been little investigated. Theorell and Åkeson [1] found 2 moles of histidine, 18 of arginine and 12 of lysine per mole of horseradish peroxidase (HRP). Tryptophan and hydroxyproline were not found to be constituents of HRP. Recently, Klapper and Hackett [2] reported the amino acid as well as carbohydrate composition of different components present in commercial HRP. The amino acid composition of Japanese radish peroxidase has been examined by Morita and Kameda [3] and that of pineapple peroxidase B by Beaudreau and Yasunobu.[4]

The present study reports the amino acid composition of crystalline cytochrome *c* peroxidase (EC. 1.11.1.5) (CcP) from baker's yeast, as elucidated mainly by the chromatographic method of ion exchange resin developed by Spackman, Stein and Moore.

MATERIAL AND METHODS

Cytochrome c *peroxidase*. Crystalline CcP was prepared by a procedure that has recently been published.[5,6] The ratio of E_{407}/E_{280} of the preparation used in this study was equal to 1.28. The protohemin content was equal to 1.759 % per unit dry weight.

Hydrolysis. Samples of the protein were hydrolyzed at 110°C in evacuated, sealed Pyrex tubes in 6 N HCl. Hydrolysis was carried out for periods of 20 and 70 h. The cooled tubes were opened and HCl was removed *in vacuo* by rotation. The final residue was redissolved in 0.2 M sodium citrate buffer, pH 2.2, and made up to 5 ml with the same buffer.

Determination of amino acids. Aliquots of 1 to 2 ml of the protein hydrolysate (containing from 1.2 to 1.5 mg of protein hydrolysate) were analyzed according to the procedure of Moore, Spackman and Stein [7] with a Beckman/Spinco 120 B amino acid analyzer.

Determination of tryptophan [8] was carried out in samples of unhydrolyzed protein by the method of Spies and Chambers.

Determination of cyst(e)ine after oxidation to cysteic acid.[9] The total cysteine plus cystine content was determined on the amino acid analyzer as cysteic acid after performic acid oxidation. The number of residues was calculated by reference to the molar quantities of amino acids stable to oxidation and to the composition of unoxidized samples.

Determination of amide ammonia. The procedure was essentially the same as that described by Laki *et al.*[10] The Conway microdiffusion technique was employed and the ammonia was determined in the amino acid analyzer. The protein was heated in 2 ml of 1 N HCl for 4 h in a stoppered tube in a water bath at 100°C. From the hydrolysates, aliquots were added to Conway vessels containing 0.01 N H_2SO_4 in the inner chamber. Saturated sodium tetraborate solution was added to the outer chamber. To obtain a value for the traces of ammonium salts present in the protein sample, ammonia of the unhydrolyzed protein was determined in the Conway vessels for different periods of diffusion time.

Table 1. Amino acid composition of crystalline cytochrome *c* peroxidase. The results are in terms of grams of amino acid residues per 100 g of protein.

Amino acid residue	Time of hydrolysis		Average or extrapolated values
	20 h	70 h	
Aspartic acid	14.22	14.10	14.27 [a]
Threonine	4.08	3.83	4.19 [a]
Serine	3.24	2.71	3.50 [a]
Glutamic acid	10.26	10.23	10.26
Proline	4.23	4.23	4.23
Glycine	3.78	3.73	3.81 [a]
Alanine	3.29	3.22	3.32 [a]
Valine	3.32	3.57	3.57 [b]
Methionine	2.25	—	2.25
Isoleucine	2.49	2.54	2.54 [b]
Leucine	7.61	7.43	7.61
Tyrosine	5.46	4.39	6.00 [a]
Phenylalanine	7.04	7.01	7.04
Lysine	7.77	7.65	7.82 [a]
Histidine	2.03	2.05	2.05
Arginine	4.12	3.99	4.17 [a]
Cysteine	0.44	—	0.44

[a] These values were obtained by extrapolation to zero time of hydrolysis.
[b] The 70 h value only.

RESULTS

Table 1 presents the analytical data obtained from 20 and 70 h hydrolysates of CcP. The release of most amino acids was complete during a 20 h hydrolysis. However, the yield of valine and isoleucine was found to increase with increasing time of hydrolysis. In the final calculation of these two amino acids the values of the 70 h hydrolysates have been used. Glutamic acid, proline, phenylalanine, and histidine appear to be stable for 70 h of hydrolysis. Decomposition with increasing time of hydrolysis occurred with threonine, serine, and tyrosine, and to a slight extent also with aspartic acid, glycine, alanine, lysine, and arginine. The concentrations of these amino acids were obtained by extrapolation to zero time of hydrolysis, using the formula [11]

$$\log A_0 = \left(\frac{t_2}{t_2-t_1}\right) \log A_1 - \left(\frac{t_1}{t_2-t_1}\right) \log A_2$$

In the calculations, A_1, A_2, and A_0 are the quantities of amino acids present after t_1, t_2, and zero hours of hydrolysis, respectively. The destruction of tryptophan was complete and therefore this amino acid was determined colorimetrically on the unhydrolyzed protein. With the "Procedure K" of Spies and Chambers,[8] a value of 3.26 % of tryptophan residues per unit weight of protein was obtained.

During the acid hydrolysis the sulfur-containing amino acids were partially decomposed or oxidized. Therefore the protein was oxidized with performic acid before hydrolysis, when cysteine and cystine were oxidized to cysteic acid and methionine to methionine sulfone. After oxidation, the protein was hydrolyzed for 20 h and the amino acid liberated analyzed in the amino acid analyzer. Cysteine, cystine, methionine, and methionine sulfoxide could not be detected in the hydrolysates of the oxidized CcP, which was assumed to indicate that the oxidation with performic acid was quantitative.

The amount of amide nitrogen of CcP was estimated by the release of ammonia at 100°C in 1 N HCl and found to be equal to 1.36 % amide residues per unit weight of protein. This corresponds to 29 amide groups per mole of CcP.

The accuracy with which the number of residues of amino acids can be estimated depends on the number of residues present in the protein. With n amino acid residues the permitted variation would be $n \pm 0.4$ and the accuracy therefore $\pm 0.4/n$.[12] A precision of $\pm$ 1 % has been reported for the automatic amino acid analyzer, which means that amino acid residues up to 40 residues per mole of protein can be calculated to the nearest integer with reliability. It can be seen from the present estimate of the composition of CcP in Table 2 that, with the above precision of the method, all amino acid residues could be calculated to the nearest integer except aspartic acid.

The average molecular weight, calculated from all the amino acid residues, was found to be 34 164, with a standard deviation of $\pm$ 584. The molecular weight of 34 144 obtained from the physical measurements conforms well with this value.[14]

A value for the partial specific volume may be calculated, according to the method of Cohn and Edsall,[15] from the weight percentages of the amino

Table 2. Composition and molecular weight of cytochrome *c* peroxidase.

Amino acid residue	Grams of amino acid residues per 100 g of protein	Minimum molecular weight [c]	Assumed number of residues per molecule M = 34 164	Calculated molecular weight [d]	Calculated number of residues for M = 34 164
Aspartic acid	14.27 [a]	806	42	33 852	42.39
Threonine	4.19 [a]	2 413	14	33 782	14 16
Serine	3.50 [a]	2 488	14	34 832	13.73
Glutamic acid	10.26	1 263	27	34 101	27.05
Proline	4.23	2 295	15	34 425	14.89
Glycine	3.81 [a]	1 497	23	34 431	22.82
Alanine	3.32 [a]	2 140	16	34 240	15.96
Valine	3.57	2 775	12	33 300	12.31
Methionine	2.25	5 831	6	34 986	5.86
Isoleucine	2.54	4 452	8	35 616	7.67
Leucine	7.61	1 486	23	34 178	22.99
Tyrosine	6.00 [a]	2 801	12	33 612	12.20
Phenylalanine	7.04	2 089	16	33 424	16.35
Lysine	7.82 [a]	1 638	21	34 398	20.86
Histidine	2.05	6 687	5	33 435	5.11
Arginine	4.17 [a]	3 743	9	33 687	9.13
Cysteine	0.44	34 354	1	34 354	0.99
Tryptophan	3.26	5 709	6	34 254	5.98
Amide ammonia	1.36 [b]	1 176	29 [b]	34 104	29.05 [b]
	90.33				
Hemin	1.76				
Totals	92.09		270	34 164 [e]	270.45

[a] These values were obtained by extrapolation to zero time of hydrolysis.
[b] These values omitted from the total.
[c] (Molecular weight of amino acid residue × 100)/percent of amino acid residue in the protein.
[d] Minimum molecular weight × the nearest integral number of residues.
[e] Average molecular weight for all residues.

acid residues and their respective specific volumes (Table 3). The calculated value of 0.727 ml/g is in satisfactory agreement with the measured value of 0.733 ml/g.[14]

Since the difference between (aspartic + glutamic — amide) and (lysine + histidine + arginine) is five acid groups, one may expect a slightly acid isoionic point for the apoprotein of CcP. A theoretical titration curve for a total of 86 ionizable groups in the protein, neglecting any end-groups, is shown in Fig. 1. For this hypothetical case an isoionic point of approximately 5.3 is obtained. The two ferriporphyrin propionic acid groups omitted from the calculations are expected to contribute somewhat to the acidic character of the protein. The isoelectric point of 4.9 measured for CcP at 0.1 ionic strength and 2°C seems therefore to conform well with the theoretically obtained isoionic point of the apoprotein of CcP.

Table 3. Partial specific volume of the apoprotein of cytochrome *c* peroxidase. The 29 amide groups have been distributed according to the ratio found for aspartic acid to glutamic acid to give 18 asparagine and 11 glutamine residues.

Amino acid residue	Amino acid residue per 100 g of protein W	$\bar{v}$	$\bar{v}\,W$
Aspartic acid	8.15	0.60	4.890
Asparagine	6.06	0.62	3.757
Threonine	4.19	0.70	2.933
Serine	3.50	0.63	2.205
Glutamic acid	6.08	0.66	4.013
Glutamine	4.15	0.67	2.781
Proline	4.23	0.76	3.215
Glycine	3.81	0.64	2.438
Alanine	3.32	0.74	2.457
Valine	3.57	0.86	3.070
Methionine	2.25	0.75	1.688
Isoleusine	2.54	0.90	2.286
Leucine	7.61	0.90	6.849
Tyrosine	6.00	0.71	4.260
Phenylalanine	7.04	0.77	5.421
Lysine	7.82	0.82	6.412
Histidine	2.05	0.67	1.374
Arginine	4.17	0.70	2.919
Cysteine	0.44	0.61	0.268
Tryptophan	3.26	0.74	2.412
Total	90.24		65.648
Partial specific volume, V		65.648/90.24 = 0.727 ml per g.	

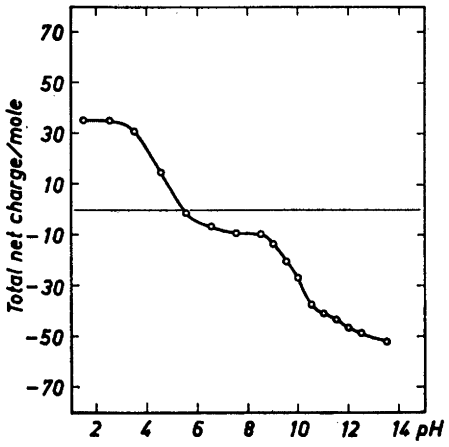

Fig. 1. A theoretical titration curve for the apoprotein of cytochrome *c* peroxidase. The calculation is based on the following pK values.[16,17]

Ionizable group	Total number in CcP	pK assumed
γ- and δ-Carboxyl	40	4.5
Imidazole	5	6.5
ε-Ammonium	21	10.0
Phenolic hydroxyl	12	10.0
Sulfhydryl	1	10.0
Guanidine	9	12.5

The terminal amino and carboxyl groups are not considered. The titrateable carboxyl groups were obtained by subtracting the amount of amide groups from the total amount of carboxyl groups.

DISCUSSION

The analytical results, expressed as numbers of residues per molecule of CcP, are generally close to integral values for most of the amino acids. The exceptions are serine, threonine, valine, isoleucine, tyrosine, and phenylalanine, of which threonine, serine, and tyrosine have been corrected for losses with increasing time of hydrolysis by extrapolating the analytical results to zero time, assuming first-order kinetics for the decomposition. However, it is possible that in the case of CcP, which is hydrolyzed as an intact protein with the hemin group attached, decompositions that do not follow a first-order course may occur. It is possible that the rate of decomposition of free amino acids may differ from that prevailling during the initial stages of protein hydrolysis. Decompositions following higher orders have been reported with threonine and serine.[18,19]

The recoveries of valine and isoleucine seem to be somewhat low, which indicates that hydrolysis for 70 h is not enough to split all peptide bonds. In the case of phenylalanine the slight deviation from the integral value remains to be explained.

The amino acid composition of CcP reveals a rather individual pattern of amino acid composition as compared with those of the plant peroxidases like horseradish peroxidase, Japanese radish peroxidase, and pineapple peroxidase B. The plant peroxidases share a property common to many plant proteins in having a low lysine content,[20] in contrast to CcP, the lysine content of which was found to be rather high. Pineapple peroxidase B, Japanese radish peroxidase, and CcP all contain tryptophan, which horseradish peroxidase does not. The tryptophan and tyrosine content of CcP is high compared with that of plant peroxidases, which explains the low ratio of E_{407}/E_{280} found for crystalline CcP.

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