ACTA CHEMICA SCANDINAVICA 4 (1950) 508 – 511

Paper Chromatography of the Amino-Acids in Horseradish Peroxidase

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In the course of studies concerning the attachment of the heme group to the protein part of horseradish peroxidase it was of importance to investigate which amino acids were present in the protein part. Theorell and Åkeson [1] in 1942 separated this protein after hydrolysis into acid, neutral and basic amino acids by an electro-dialytic micro-method. In the alkaline fraction they found histidine, arginine and lysine.

We used their technique and analysed the three fractions separately by paper chromatography using the ascending method introduced by Williams and Kirby [2]. Munktell OB filterpaper 48 × 48 cm was used, cut to a size of 45 × 45 cm and the solution of the sample was placed in one corner of the sheet, 5 cm from each edge, by means of a micropipette (ordinary type or the type described by Linderstrøm-Lang [3].) A Petri-dish with diluted ammonia was placed underneath the paper when applying acid solutions. The volumes ranged between 2 and 200 μl, but no more than 5 μl were put on the paper at the same time. A stream of compressed air was used to dry the paper after each application. The sheet was now formed into a cylinder and loosely sewn together by white twine. It was placed into a glass cylinder 50 cm high and 25 cm wide filled with a mixture of phenol-water 4 : 1 (4), some drops of 35 % ammonia and a few mg of 8-hydroxy-quinoline to prevent Cu-fronts on the paper. A beaker containing about 100 mg NaCN was placed on the bottom.* The glass cylinder was sealed with a 5 mm thick glass plate, tightened with "plastiline" and placed in a thermostat at 37° over night. At this temperature, the front of the solvent had reached the top of the paper after 10—12 hours, whereas at room temperature (24°) about 24 hours were needed.

* Complete saturation of the solvents is unnecessary in practice and may be even impracticable on account of eventual emulsification [5].

The sheet was cut up and dried by an air stream at room temperature. After cutting away a 5 cm wide strip at the top of the sheet (including the slightly colored front of the phenol) it was again sewn into a cylinder and turned 90°. The second run was made in a collidine-lutidine-water mixture 1 : 1 : 1 under the same conditions as the phenol run but without any additions to the solvent. After 10—12 hours the solvent had reached the top of the paper.

The sheet was dried as before and sprayed with a butanol solution saturated with water and containing 0.1 % ninhydrin and 1 % acetic acid [6]. The solution was applied with compressed air, using a sprayflask, made by "Kifa", Stockholm. After hanging in the thermostat at 37° for a few hours the paper distinctly showed the characteristic spots of the amino acids. Heating to 105° was unfavorable as Dent has already stated [7].

The analyses were first made with samples and mixtures of synthetic amino acids dissolved in 75 % EtOH (if necessary with addition of very small amount of conc. HCl) and the R_f-values measured. The following results were obtained:

Table 1. R_f-values of amino acids used.

Amino acid	Source	HCl µl/ml	$R_{f(37°)}$ in phenol	$R_{f(37°)}$ in collidine
1. D,L-Alanine	*	0	.55	.17
2. L-Arginine-HCl	Merck	0	.77	.01
3. D,L-Aspartic acid	Merck	30	.18	.17
4. L-Cysteic acid	**	0	.07	.30
5. D-Glutamic acid HCl	*	0	.30	.17
6. D,L-Glycine	Shering-Kahlbaum	0	.37	.12
7. L-Histidine-HCl	Hoffman-La Roche	15	.64	.15
8. L-Isoleucine	—»—	0	.71	.40
9. D-Leucine	Schuchardt	0	.71	.40
10. L-Lysine-HCl	Merck	0	.61	.01
11. D,L-Methionine	Eastman-Kodak Co.	0	.72	.41
12. D,L-Norleucine	*	0	.71	.40
13. L-Hydroxyproline	Schuchardt	0	.59	.18
14. D,L-Phenyl-alanine	*	0	.73	.47
15. L-Proline	*	0	.75	.20
16. D,L-Serine	Merck	20	.33	.13
17. D,L-Threonine	»	0	.47	.17
18. L-Tyrosine	Hoffman-La Roche	50	.55	.49
19. L-Valine		0	.69	.31

* Obtained by courtesy of Dr A. Wretlind, Karolinska Institutet, Stockholm.

** Prepared according to Gortner and Hoffman [8].

The solvents were purified in the following way:

1. *Lutidine.* A mixture of the 2,4- and 2-5-isomers with b. p. $155-160°_{760}$ was obtained from "Yorkshire Tar Distillers Ltd., Cleckheaton, Yorks". Further treatment proved to be unnecessary.

2. *s-Collidine.* The purified product was also perchased from "Yorkshire Tar Distillers Ltd". It was treated with bromine according to Consden *et al.*[9] and distilled *in vacuo*. B. P. 169—171°. The product was slightly yellow and darkened very slowly if at all.

3. *Water.* Redistilled in all-glass apparatus.

4. *Phenol.* U. S. P., Baker Co., was distilled *in vacuo* according to Draper and Pollard [10] but using Zn-powder instead of Al. B. P. 182°.

5. *n-Butanol.* The product obtained from "Kebo AB", Stockholm, was fractionated on a Widmer-column and the fraction boiling between 116—117° was used.

For the analysis of the horseradish peroxidase the following procedure was used:

1. 1 ml 1.4 % dialysed sample of the peroxidase, crystallized according to Theorell [11] and with a P. N. 1020, was hydrolyzed by refluxing with 2.95 ml 37 % HCl and 1.5 ml water (= 20 % solution) for 24 hours.

2. Humin substances were removed by filtration.

3. The hydrolysate was evaporated *in vacuo* after addition of two drops of 8 *N* H_2SO_4 to get rid of Cl^-.

4. Electrodialysis in the microapparatus of Theorell and Åkeson [1]. Each fraction was run twice.

5. Each fraction was evaporated to 0.1—0.4 ml *in vacuo*.

6. Small volumes corresponding to about 0.5 mg total content of amino acids were applied on the paper as described before.

For tryptophane analysis a sample of the peroxidase was hydrolysed in alkali and treated according to Steers and Sevag [12]. A 10 mg hydrolysate gave no color development, whereas 10 γ tryptophane (Merck) gave positive reaction.

The following results were obtained:

Table 2. Amino-acids found in horseradish peroxidase.

Anode (acid fraction)			Middle compartment (neutral fraction)			Cathode (alkaline fraction)		
Acid	Color	Present	Acid	Color	Present	Acid	Color	Present
asp.	purple	+	ala.	purple	+	arg.	dark purple	+
cys.	blue	+	gly.	purple	+	his.	grey purple	+
glu.	purple	+	hypro.	—	—	lys.	purple	+
			leu.	purple	+			
			met.	purple	+			
			phe.	grey purple	+			
			pro.	yellow	+			
			ser.	purple	+			
			thr.	purple	+			
			try.	—	—			
			tyr.	grey purple	+			
			val.	purple	+			

Unidentified spots were observed twice in the cathode fraction. One had R_f 0.67 in phenol and 0.30 in collidine, the other R_f 0.77 in phenol and 0.41 in collidine.

Abbreviations of the amino acids according to Brand and Edsall [13], cys = cysteic acid.

Work on the quantitative determination of the amino acids in horseradish peroxidase protein is being continued.

SUMMARY

A modification of the ascending method of paper chromatography according to Williams and Kirby [2] has been described. With the aid of this technique the amino acid composition of horseradish peroxidase was investigated. The usual amino acids were identified except tryptophan and hydroxyproline. Two unidentified spots were found in the cathode fraction of the electrodialyzate.

This work was aided by a grant from *Stiftelsen Therese och Johan Anderssons minne* for which the authors wish to thank.

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Received January 31, 1950.