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Four Isoperoxidases from Horse Radish Root

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Four peroxidases have been isolated from pooled horse radish roots in the form of homogeneous proteins. One of them is highly acid (I), one is neutral (III), and two are strongly basic (V, VI). Their isoelectric points well reflect the amino acid compositions.

One of the basic peroxidases (VI) might be formed from III by the loss of 40 amino acids, including 14 acid but no basic residues. The carbohydrate content is of the same magnitude in I, III, and VI whereas the other basic peroxidase has about half the carbohydrate content. This peroxidase also shows a low-spin spectrum whereas the other three give high-spin forms.

The acid peroxidase contains half as much arginine and methionine as the other three peroxidases and it gives one third of the activity with guaiacol as the hydrogen donor.

Plant peroxidases have two properties in common: they occur in most, perhaps all, parts of the plants, and their hydrogen donor specificity is low. Multiple components of peroxidases have been found in a number of plants such as horse radish,[1–5] Japanese radish,[6,7] broad bean leaves,[8] sweet potato,[9] wheat germs,[10,11] pea,[12,13] petunia,[14] and turnip.[15,16] A preference of one or more peroxidase fractions for a certain part of a plant [12,17–19] as well as developmental differentiations [13,20,21] have been described.

The "isoperoxidases" have been separated electrophoretically, but their isolation in the pure form is necessary to elucidate what structures constitute the difference. The access to homogeneous peroxidase preparations might also shed light on the hydrogen donor requirements of these enzymes.

Before the chromatographic era Theorell [22] isolated two components of horse radish peroxidase, I and II. II was neutral with a high carbohydrate content whereas I was basic and essentially carbohydrate-free. The protein moieties appeared identical as judged from the analyses of some amino acid.[23] Klapper and Hackett [5] separated into three acid fractions a commercial preparation of horse radish peroxidase by means of starch gel electrophoresis and ion exchange chromatography. The spectra, molecular weights, amino acid compositions, and activities (two hydrogen donors) were essentially identical. Shannon *et al.*[3] analyzed five of seven isolated fractions for amino acids and carbohydrates and described their spectral properties. Japanese

radish peroxidases *a* and *c* (of types A and C, *vide infra*) differed [7,24–26] in molecular weights (54 500 and 41 500), I.P. (3.5 and≈11), and amino acid compositions, but gave identical *RZ* values (3.65 and 3.66).

Morita [27] classified plant peroxidases on the basis of their spectra. Type A shows absorption maxima at 403, 500, and 640 nm in the oxidized form, and at 438 and 560 nm in the reduced form with a slight decrease in the Soret band absorbancy upon reduction. Type B gives maxima at 414 and 530 nm and at 428, 534, and 563 nm, respectively, with a slight increase of the Soret band upon reduction. Type C has its maxima at 419, 540 nm, and 425, 563 nm with a drastic increase of the Soret band upon reduction.

For some time we have been using a procedure for the isolation of horse radish peroxidase which yields products which disagree somewhat with the above results. Four main fractions have been isolated and characterized as different proteins.

MATERIALS AND METHODS

All isolation steps, including column operations and concentrations, were performed in the cold room at about +4°C.

Carboxymethyl cellulose (CMC) was prepared according to Peterson and Sober.[28] The Sephadex gels and ion exchangers were purchased from Pharmacia (Uppsala and Umeå, Sweden) and conditioned as prescribed by the manufacturer. Pharmacia columns were used for recycling. Protein solutions were concentrated on a Diaflo ® membrane (retaining mol. weights above 10 000) under N_2. All peroxidase spectra were measured in 0.1 M phosphate, pH 7.0.

Hematin was assayed as pyridine hemochrome [29] and expressed as ferri protoporphyrin Light absorption spectra were determined in a Beckman DU spectrophotometer, operated at narrow slits and calibrated against a Holmium filter.

For dry weight determinations 0.1—0.5 ml was kept at 105°C for 2 h or to constant weight.

Starch gel electrophoresis was run for 2 h at 20 V cm^{-1} with 75 mM tris-citrate, pH 8.65, in the gel and 300 mM borate, pH 8.2, in the electrode vessels.[30] The starch powder was purchased from Connaught Medical Research Laboratories, Toronto.

Disc electrophoresis in polyacrylamide (PA) gels was run in either of two systems: (a) according to Ref. 31; (b) top buffer 5 tris, 40 Gly, pH 8.3; spacer gel 1.25 % PA (riboflavine), 60 tris, 32 H_3PO_4, pH 6.9; bottom gel 7.5 % PA (persulphate), 375 tris, 50 HCl, pH 8.9; bottom buffer 121 tris, 60 HCl, pH 8.1 (all concentrations in mM).

Both types of electropherograms were stained with aniline blue black or benzidine-H_2O_2.

Isoelectric focussing was performed in the LKB column 8101, 110 ml (LKB, Stockholm, Sweden) with 1 % Ampholine according to LKB's suggestions and sucrose for the density gradient.

Amino acids were determined by a Beckman instrument model 120C with an Infotronics integrator CRS-100A after hydrolysis for 24 and 72 h with 6 M HCl in evacuated and sealed tubes. Norleucine (Calbiochem AG, Lucerne, Switzerland) was dried over silica gel and used as internal standard. Ammonia and basic amino acids were separated on the sulphonated styrene co-polymer Beckman PA-35; other amino acids and hexosamines were separated on the similar Beckman PA-28 resin. The values in Table 5 are extrapolated to zero time for Thr, Ser, Tyr, NH_3, and Glc·NH_2; for Pro and Leu the mean value, and for the other amino acids the maximum value has been used.

The carbohydrate contents were determined by the anthrone method as modified by Shields and Burnett [32] with a 1:1 mixture of D-galactose and D-mannose as reference.

Activities were measured according to Maehly *et al.* [33] and expressed as moles of "tetraguaiaco-quinone" formed per mole of hematin per minute. ε_{mM} for the oxidation product of guaiacol was taken as 26.6 at 470 nm.

RZ is defined as A_{403}/A_{280} for all components.

ISOLATION PROCEDURE

Extraction and initial purification. Peel from well rinsed horse radish roots were scraped off manually in flakes with a thickness of 1 mm or less. The description refers to a quantity of 25 kg. The peel was extracted with occasional stirring for 2 × 4 h with 1+0.5 l of precooled, deionized water. The "brei" was poured into a plastic cylinder, height:diameter 4:1, with a gauze-covered perforated bottom. The first turbid liters to leave the cylinder were returned to the column which then delivered a nearly clear filtrate (volume *a* liters). Solid ammonium sulphate was added to give 40 % saturation (0.25 *a* kg), and the solution was stirred for a few hours. The precipitate was removed in the centrifuge and another 0.41 *a* kg of ammonium sulphate was added. This quantity would not saturate water, but left a tiny amount of undissolved salt in the extract. The batch was stirred overnight, and the precipitate collected by centrifugation and dialyzed for 2 days against running tap water. A grayish precipitate was removed, and one volume of 95 % ethanol, precooled to −10°C, was slowly added with stirring. The precipitate was collected by centrifugation and discarded, and another 3 volumes of ethanol were added as above. This precipitate was collected in the centrifuge and dialyzed against 4 mM acetic acid, preadjusted to pH 4.70 at 4°C with NaOH. During this dialysis a slight precipitate appeared which was carefully removed (13 000 *g*, 60 min).

Chromatography on CMC. The solution was applied without preceding concentration onto a CMC column, preequilibrated with the above buffer. The column should not be oversized, 130 × 30 mm being well enough. A variety of elution procedures yielded essentially identical fractions, and a stepwise elution was adhered to (Fig. 1). Some material

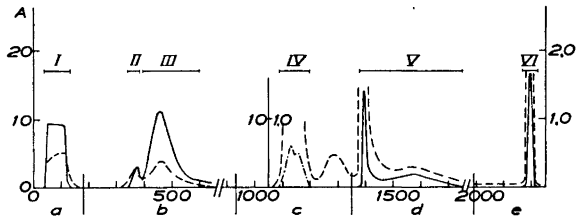

Fig. 1. Chromatography of crude HRP (*RZ* 1.62) on CMC, preequilibrated with 4 mM acetate, pH 4.70. - - - - A_{280}, ——— A_{402}, ·— ·— A_{610}. Buffers for elution: a) 4 mM acetate, pH 4.70; b) 30 mM acetate, pH 4.70; c) 30 mM acetate, pH 5.70; d) 30 mM sodium phosphate, pH 7.35; e) 50 mM disodium phosphate.

was only slightly retained by the column and passed straight through (fraction I). Fraction II appeared somewhat irregularly and may represent the tail of I. Fraction III usually showed some tailing, but gradient elutions resulted in poor separation from the next fraction. When III had left, the column was from the top reddish-brown, blue, and colourless. The blue protein (fraction IV), which usually appeared as two blue bands on the column, was eluted with 30 mM acetate, pH 5.70. The reddish fraction V migrated slowly with this buffer but was eluted with phosphate at a higher pH; some tendency toward its separation into subfractions appear regularly. Fraction VI is defined as the hemoprotein eluted with 50 mM Na_2HPO_4. No cytochrome *c* was observed on the column. All fractions were dialyzed against distilled water or the buffer to be used next, and concentrated.

The ratios between the amounts of the various hematin-containing fractions in terms of Soret band absorption are given in Table 1 together with some properties of the fractions.

Further purifications. Fraction I was dialyzed against 20 mM acetate, pH 5.85, and put on a DEAE Sephadex A-50 M column (Fig. 2). The three fractions I a–c gave *RZ* 3.6, 4.2, and 3.0, all with their Soret band maxima at 403 nm. When fraction Ib, which accounted for the largest part, was rechromatographed in the same system, the *RZ* rose to 4.3.

Table 1. Properties of peroxidase fractions from CMC columns. ε was determined on the basis of hemin content. The activity is expressed as mmole of "tetraguaiaco-quinone" per μmole of hematin per minute.

Fraction	Rel. amt. % from A_{403}	A_{403}/A_{280} RZ	Soret band ox ε mM	ox nm	red ε mM	red nm	Activity
I	23	2.2	107	405	97	441	8
II	3	1.0	131	403	109	437	37
III	50	3.1	100	403	89	437	28
V a	4	0.3	140	403	99	431	26
V b	4	1.0	138	415	154	434	37
V c	4	1.0	109	410	113	431	28
VI	12	0.6	95	404	82	438	26

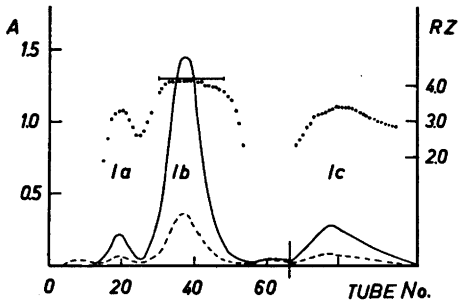

Fig. 2. Chromatography of fraction I on DEAE-Sephadex (150 × 18 mm), pre-equilibrated with 20 mM acetate, pH 5.85. Fractions Ia and Ib were eluted with this buffer, Ic with 60 mM acetate, pH 5.30. ---- A_{280}, ——— A_{403}, ···· A_{403}/A_{280}.

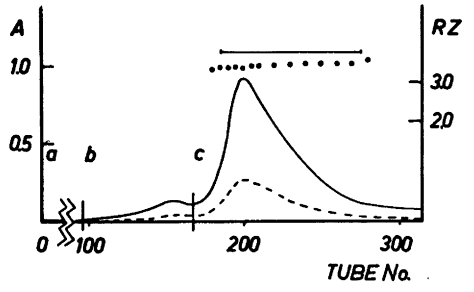

Fig. 3. Chromatography of fraction III SE-Sephadex (50 × 20 mm), preequilibrated with 10 mM acetate, pH 4.70. Elution with a) this buffer, b) a linear gradient 10—30 mM, and c) 30 mM acetate buffer, all of pH 4.70.

Fraction III was homogeneous on Sephadex G-75 (900 × 26 mm, 50 mM sodium phosphate in 340 mM NaCl, pH 7.5) with $RZ = 3.4$. DEAE Sephadex A-50 M (100 × 19 mm, 30 mM Tris—HCl, pH 8.25) disclosed a small brown subfraction. Its separation could not be achieved on CMC with any gradient or column dimension. Finally SE-Sephadex separated two fractions (III a, b) (Fig. 3). After rechromatography in the same way III b gave $RZ = 3.47$.

Fraction IV, which was intensely blue, gave a positive reaction for copper with dibenzoyl dithiocarbamate. So far it has not been identified with any known copper protein. It has tentatively been namned "Umecyanin", and its purification and properties will be described elsewhere.[34]

When rechromatographed on CMC, fraction V was resolved into a brown Va and two reddish Vb and Vc (Fig. 4a). When chromatographed a third time on CMC, Vb gradually changed colour to brown and was partly eluted as Va. Vc split up into two subfractions. Thus fractions Vb and c are labile. Vb was finally purified on SE-Sephadex (Fig. 4b). After this step it was homogeneous on Sephadex G-75 although its colour continuously deteriorated.

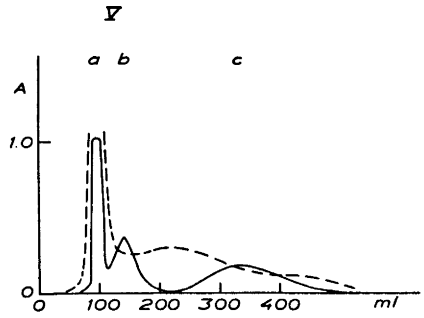

Fig. 4a. Rechromatography of fraction V on CMC (110×18 mm). Elution with a linear gradient 10–30 mM phosphate, pH 7.35.

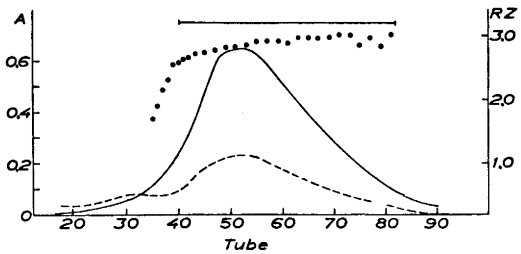

Fig. 4b. Chromatography of fraction Vb ion SE-Sephadex (60×17 mm), pre-equilibrated with 10 mM acetate, pH 4.70. Sample dialyzed against this buffer and eluted with a gradient 10–60 mM acetate, pH 5.70. ----A_{280}, ——— A_{402}, ····A_{402}/A_{280}.

Fraction VI was rechromatographed on CMC with a gradient 10–35 mM sec phosphate, which removed some non-hematin, UV-absorbing material. Then this peroxidase was homogeneous on Sephadex G-75 (900×15 mm, 20 mM acetate, pH 5.85).

TESTS FOR HOMOGENEITIES

The homogeneities of the four major fractions Ib, IIIb, Vb, and VI were tested in five ways.

a. Repetition of the last step on the cellulose ion exchanger brought about no increase in *RZ* in any one of the four peroxidases.

b. Gel filtration on Sephadex G-75 (60 mM sodium phosphate in 340 mM NaCl, pH 7.4, for Ib and IIIb, 60 mM acetate, pH 5.70, for Vb, and 20 mM acetate, pH 5.85, for VI) revealed no inhomogeneity or change in *RZ* during the elution.

c. In starch gel electrophoresis at pH 8.2 Ib and Vb appeared as homogeneous substances although Vb showed heavy tailing. A faint component,

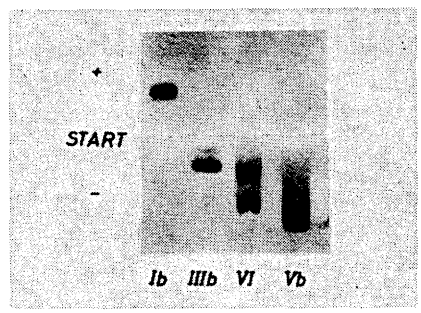

Fig. 5. Starch gel electrophoresis of the four main fractions. 0.1 % Amido Black in 7 % HAc, decolourization with the same solvent.

slightly more acid than the bulk of the fraction, was barely discernible in IIIb; it showed peroxidase activity but did not correspond to any other fraction (Fig. 5). Peroxidase VI was resolved into two components, both of which gave strong peroxidase reaction in the gel. Neither fraction corresponded to any other peroxidase in the preparation. The primary chromatogram on CMC (Fig. 1) sometimes gave two subfractions of VI, which, however, failed to reappear on the starch gel.

d. Isoelectric focussing of Ib, IIIb, and VI revealed single fractions with symmetrical peaks when examined at 403 and 280 nm (Fig. 6). VI also gave single peaks at both wavelengths but with a broad and slightly displaced 280-maximum; this effect may have been caused by the high pH and proximity to the cathode solution.

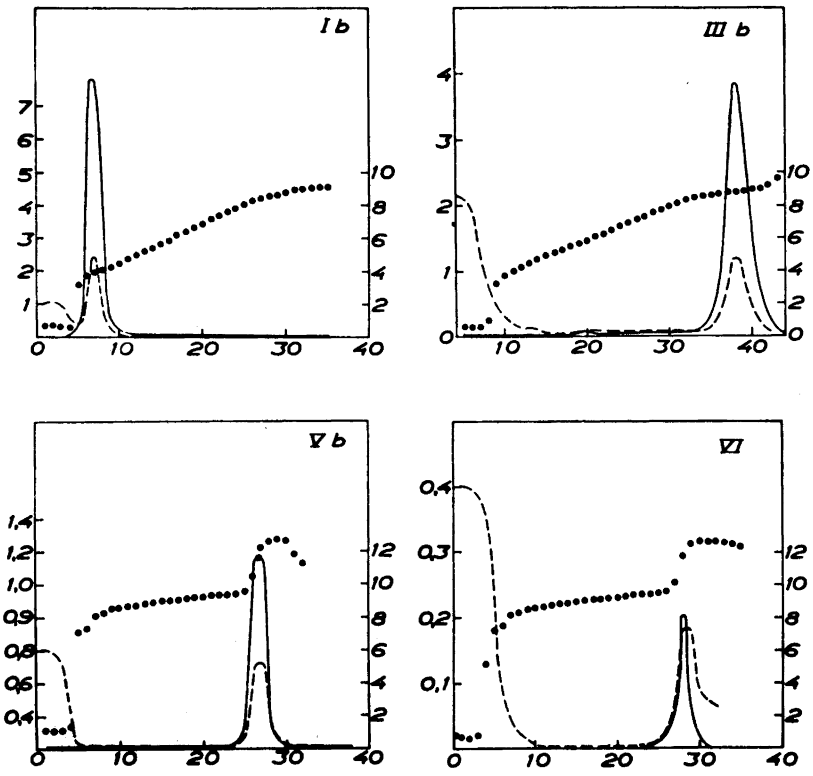

Fig. 6. Isoelectric focussing of the four fractions. 24—36 h, +10°C, 300 V. - - - - A_{280}, ——— A_{404}, ····pH. Left ordinate absorbancy, right ordinate pH. Abscissa tube number.

e. Polyacrylamide gel electrophoresis revealed single components in Ib, IIIb, and VI (Fig. 7). In Vb two broad zones appeared, one of which included a distinct band. The zones were extended so far that they probably should be considered to be artefacts.

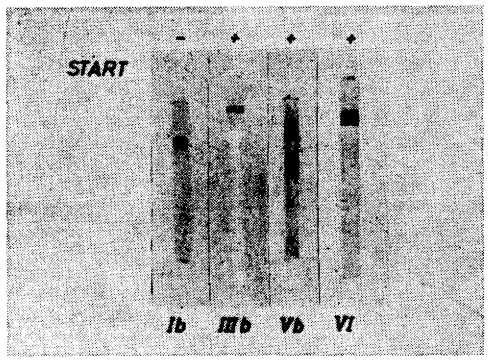

Fig. 7. Polyacrylamide gel electrophoresis of isolated fractions. Ib was run in system a, and the other three in system b (of text). 3 h, 3 mA cm^{-2}, no indicator during run.

The homogeneity tests are summarized in Table 2. All fractions are homogeneous by the criteria of the preparative procedures and the isoelectric focussing. Both gel electrophoretical methods showed the acid component Ib to be homogeneous at slightly alkaline reaction. IIIb was homogeneous on polyacrylamide gel and gave a very faint tail fraction on starch gel. Vb was homogeneous on starch gel, but gave two zones on polyacrylamide gel, whereas VI showed the reversed behaviour with two zones on the starch gel. Adsorption artefacts cannot be excluded, and there is no real evidence for heterogeneity of either preparation.

Analyses of peroxidases. The four fractions were analyzed as summarized in Tables 3 – 5.

Table 2. Tests for homogeneity. + denotes homogeneity, ( ) heterogeneity.

	Ib	IIIb	V	VI
Chromatography on ion exchanger	+	+	+	+
G75 filtration	+	+	+	+
Starch gel electrophoresis	+	+	+	( )
Isoelectric focussing	+	+	+	+
Polyacrylamide electrophoresis	+	+	( )	+

DISCUSSION

Four peroxidatically active fractions were obtained as homogeneous proteins, but several minor peroxidase fractions were removed during the isolation. Strikingly, three of the four main fractions ("b") appeared with small contaminants ("a"), removed only in the last step; the a's may derive from the b's. The amino acid analyses definitely revealed the four peroxidases to be different proteins.

As evidenced by the hemin analyses and the sum of their components, Vb and VI are smaller molecules than Ib and IIIb, and it may be questioned

Table 3. Composition of peroxidases.

Component	Hemin %	Mol. weight [a]	Mol. weight [b]	Carbohydrate %	I.P. [c]
1 b	1.42	45 900	40 200	16.2	4.0
III b	1.42	45 900	41 600	16.4	8.8
V b	1.79	36 400	33 200	7.2	> 11
VI	1.77	36 800	35 800	16.2	> 11

[a] From the hemin content.
[b] From the sum of amino acids, carbohydrates, and hematin, corrected for yield in hydrolysis but excluding tryptophane and cystine.
[c] From the isoelectric focussing.

Table 4. Light absorption of peroxidases in the protein and Soret band regions. 0.1 M sodium phosphate, pH 7.0.

Fraction	ε mM[a] 280 nm	ε mM[a] 403 nm	$\frac{A_{280}}{A_{250}}$	*RZ*
1 b	27.8	115.0	0.93	4.13
III b	28.7	99.8	1.12	3.47
V b	36.8	95.0	0.92	2.58
VI	40.8	122.9	1.00	3.01

[a] On the basis of hemin content.

Table 5. Amino acid analyses. "mg dry weight" includes hemin and carbohydrates. Quantities taken for hydrolysis were 0.828, 1.016, 1.391, and 1.544 mg from fractions I b, III b, V b, and VI.

Amino acid	μmole per mg dry weight: I b	III b	V b	VI	μmole per μmole of hemin: I b	III b	V b	VI
Asp	1.019	1.003	0.994	1.089	46.6	46.7	36.2	39.4
Thre	0.542	0.545	0.590	0.545	24.8	25.4	21.5	20.0
Ser	0.921	0.557	0.580	0.762	42.2	25.9	21.2	28.0
Glu	0.476	0.445	0.479	0.383	21.8	20.7	17.5	13.7
Pro	0.269	0.370	0.349	0.373	12.3	17.2	12.7	13.7
Gly	0.557	0.362	1.797	0.502	25.5	16.9	29.0	18.4
Ala	0.625	0.478	0.547	0.727	28.6	22.3	20.0	26.7
Val	0.301	0.343	0.696	0.443	13.8	16.0	25.4	16.2
Met	0.041	0.084	0.098	0.092	1.9	3.9	3.6	3.5
Ile	0.332	0.284	0.371	0.340	15.2	13.4	13.6	12.5
Leu	0.706	0.738	0.588	0.675	32.3	34.4	21.4	24.7
Tyr	0.070	0.118	0.044	0.108	3.2	5.5	1.6	3.9
Phe	0.415	0.434	0.321	0.445	19.0	20.2	11.7	16.3
Lys	0.118	0.132	0.130	0.176	5.4	6.2	4.7	6.5
His	0.064	0.064	0.098	0.073	2.9	3.0	3.6	2.7
Arg	0.194	0.395	0.569	0.529	8.9	18.4	20.7	19.4
					304.4	295.9	264.4	265.6
NH_3	1.582	1.316	0.747	0.766	72.5	61.3	27.2	28.1
Glc·NH_2	0.318	0.380	0.018	0.047	14.6	17.7	1.3	3.0
Gal·NH_2	0	0	0	0				

whether they represent fragments of the bigger molecules. The higher number of residues per hemin of Val, Met, and Arg in Vb excludes the possibility of Vb being a fragment of Ib, and likewise the content of Ser, Met, and Arg in VI excludes a conversion of Ib into VI. Gly and Val are markedly higher in Vb than in III, and the composition of Vb deviates in general from that of the other fractions. A degradation of IIIb into VI cannot, however, be definitely excluded. No amino acid is significantly more abundant in VI than in IIIb, but some residues are present in equal number and some have decreased markedly ($Asp_{3.7}$ $Thre_{5.4}$ $Glu_{7.0}$ $Pro_{3.5}$ $Leu_{9.7}$ $Tyr_{1.6}$ $Phe_{3.9}$). Thus, if a conversion takes place, every third amino acid lost would be acid. There would also be a loss of 14.7 Glc·NH_2, but the very large correction for the decay of hexosamines under the present conditions of hydrolysis renders the comparison indefinite. To summarize, the amino acid compositions show Ib, IIIb, and V to be different proteins whereas VI may be the hematin-containing residue of IIIb after the removal of a strongly acid fragment. Upon redution the spectral properties of IIIb and VI parallel each other.

(Asp + Glu) is high and equal in Ib and IIIb, and lower but still equal in Vb and VI. In IIIb, Vb, and VI, His, Lys, and Arg are equally abundant, whereas the content of Arg is only half as high in Ib. The difference (Asp + Glu) − (His + Lys + Arg) hence becomes largest in Ib (51.2), medium in IIIb (39.8), and lowest in Vb (27.4) and VI (24.5). In accordance with these values, amide-N disregarded, Ib has low (4.0), and Vb and VI very high I.P. (>11), whereas IIIb falls between. The content of Glc·NH_2 is highest in the two peroxidases which are rich in Asp + Glu.

The *RZ* value of Ib is significantly higher than that of IIIb. Their molar absorptions are equal at 280 nm, whereas the IIIb absorption is higher at the Soret band maximum, 403 nm. The same situation was found for two fractions of lactoperoxidase,[35] but in this case the absorption curves in UV were superimposable; this is not the case with Ib and IIIb (Table 4).

RZ of fraction Vb is low. During the isolation the original reddish colour of this fraction gradually turned to brown. Simultaneously its Soret band migrated from about 420 to 403 nm. It may be that this shift was incomplete at the time of spectral investigation which would cause a lower *RZ* value; the Soret band was somewhat broader than usual. The spectral changes upon reduction are the same as those described by Morita for peroxidase type B or C. Yamazaki *et al.*[36] tentatively identified a cyanide group attached to type B peroxidase hematin and managed to remove it by means of PCMB. Its removal converted the spectrum from low to high spin form.

The previous observation [2] that the most acid peroxidase is less active at pH 7 than the other fractions has been confirmed. All peroxidases with the higher activity (IIIb, V, and VI) towards guaiacol contain nearly equal amounts of arginine (18.4 − 20.7 residues) and methionine (3.5 − 3.9), whereas Ib has fewer residues (8.9 and 1.9); no such parallel between activity and composition exists for the other basic amino acids. In these preliminary measurements of the overall activity IIIb and VI did not differ greatly. Thus, if the former has given birth to the latter by losing one or several acid fragments, such a change has not influenced the activity in this test.

The fractions isolated by Klapper and Hackett [5] by starch gel electrophoresis would, according to their migration directions, all be classified as subfractions of I, in our terminology. Their amino acid compositions and activities did not vary significantly.

Shannon *et al.*[3] analyzed five fractions, three closely related A-fractions, one B, and one C. As judged from the relative amounts of Arg and Met their A's correspond to our I's, and their B and C to our III. This is also in agreement with the spectral properties to the extent they have been studied by us. The relative amounts of Arg, His, Val, and Tyr and the spectral properties show that Vb has no correspondence in Ref. 3. Moreover, Shannon *et al.* found a uniformly high carbohydrate content (18 %) with a low hexosamine content. The use of a relatively strong acid buffer [3] (100 mM acetate, pH 4.4) may have converted the reddish hemoprotein into its brown form.

The Japanese radish peroxidase *a* resembles closely our type I as judged from its spectrum, I.P., amino acid composition, and hexosamine content, whereas it is distinguished from IIIb by amino acid composition and I.P. On the other hand there is, on the same basis, a close relationship between Japanese radish peroxidase *c* and Vb; their spectra, too, agree. Our type III seems to be absent, however.

In summary, several authors have isolated by chromatographic methods a strongly acid peroxidase (I, A, *a*), which may be commonly distributed. There is less uniformity among the neutral and basic fractions, which may depend either upon the mode of preparation or the starting material.

Heterogeneity is frequent among hemoproteins and in plant proteins. Desamidations do occur as well as serial degradations of the carbohydrate moieties. In the present case, partial degradations – with one possible exception – do not account for the occurrence of the main peroxidases but may well be responsible for the "a"-fractions. On the contrary, available results reveal the existence of three or four peroxidase proteins with different primary structures. This variety can be interpreted in three ways:

a) The material is genetically inhomogeneous. This would easily be tested, but the plant is unaccessible for genetic studies since it is sterile.

b) The enzymes are localized to different parts of the plants, and for some reason the tissues require different proteins for identical functions.

c) The enzymes have a hydrogen donor, *i.e.* substrate, specificity. This possibility may be linked to the distribution in the plant.

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