Isolation of a Peroxidatically Active Product from the Peptic Digest of Ox-Liver Catalase and some of its Properties

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1. A homogeneous peroxidatically active product has been isolated from a peptic digest of ox-liver catalase. 2. The nitrogen and the iron contents of the 'active product' are 15·3% and 0·21% respectively. 3. The $S^0_{20,w}$ and molecular weight of the 'active product' are 2·6s and 27500 respectively. 4. The 'active product' contains 248 amino acid residues/mol. assuming a mol.wt. of 27500. 5. The properties of the 'active product' and catalase are compared and the relationship between their structures and enzyme activity is discussed.

Waentig & Steche (1913) observed that catalase was inactivated by trypsin and by the gastric juice of crabs, but not by weakly acid pepsin. Anan (1958*a*) reported that the inactivation of catalase by trypsin and pepsin is followed by an increase in its peroxidatic activity. Anan (1958*b*) isolated a 'pepsin-modified catalase', studied its catalytic properties, and gave limited information on its chemical and physical properties.

Radhakrishnan, Raghupathy & Sarma (1963) found that the catalatic activity of catalase was decreased on degradation with α-chymotrypsin and trypsin, whereas its peroxidatic activity was increased after digestion with α-chymotrypsin and diminished slightly after proteolysis with trypsin. In the present paper we report on the influence of pepsin on crystalline ox-liver catalase and describe the isolation of a peroxidatically active product from the peptic digest. The properties of the active product and catalase are compared and the relationship between their structures and biological activities is discussed.

MATERIALS AND METHODS

Materials. Water, redistilled in Pyrex-glass apparatus, and only AnalaR reagents were used. Salt-free crystalline pepsin and crystalline ox-liver catalase were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. The catalase behaved as a homogeneous protein on paper electrophoresis and during ultracentrifugation.

Peptic digestion of catalase. A 1% (w/v) solution of catalase was adjusted to pH 4·0 with 0·05N-HCl. Of this, 3ml. portions were taken in two stoppered test tubes and equilibrated at 30° for 30min. To one tube (test) was added 0·1ml. of a 1% (w/v) solution of pepsin at pH 4·0, and the other tube (control) received 0·1ml. of 0·05N-HCl. The mixtures were incubated at 30° for 24hr. in the presence of a few drops of redistilled toluene.

Determination of catalase activity. The catalatic activities of the test and control samples were measured at intervals by the titrimetric method of Euler & Josephson (1927) as described by Radhakrishnan *et al.* (1963). Catalatic activity was calculated as ml. of 0·1N-$KMnO_4$ equivalent to the H_2O_2 consumed/min./mg. of protein nitrogen. Relative activities are expressed as percentages of activities of control samples.

Determination of peroxidatic activity. This was measured by the pyrogallol method of Willstätter & Stoll (1917) as modified by Sumner & Gjessing (1943). The purpurogallin formed by the action of the digestion mixture during 5min. at 25° in the presence of H_2O_2 and pyrogallol was measured in a Klett–Summerson photoelectric colorimeter, and a calibration curve was obtained with 'Sigma grade' purpurogallin. Peroxidatic activity was calculated as mg. of purpurogallin formed/mg. of protein nitrogen.

Rate of proteolytic degradation. Samples withdrawn at intervals from the digestion mixture were treated with trichloroacetic acid to give a final concentration of 10% (w/v). The precipitate was collected by centrifugation and washed with trichloroacetic acid. Nitrogen in the precipitate was determined by a micro-Kjeldahl procedure followed by direct nesslerization (Koch & McMeekin, 1924).

Estimation of iron. Iron was determined colorimetrically by the αα′-bipyridyl method of Kitzes, Elvehjem & Schuette (1944) after digesting the samples with a mixture of conc. HNO_3 and conc. $HClO_4$.

Paper electrophoresis. This was carried out as described by Radhakrishnan & Sarma (1964). The protein bands were located on the paper strips by staining with 0·1% (w/v) bromophenol blue in ethanol saturated with $HgCl_2$.

Isolation of a peroxidatically active pepsin-degraded product from a peptic digest of catalase. (*a*) Digestion of catalase with pepsin. A 300ml. portion of a 1% (w/v) solution of ox-liver catalase at pH 4·0 was incubated at 30° with 10ml. of a 1% (w/v) solution of crystalline pepsin at pH 4·0 and about 0·1ml. of redistilled toluene. After 24hr. the pH of the mixture was adjusted to 7·0 with dilute NaOH.

(*b*) Ammonium sulphate fractionation of the peptic digest of catalase. The neutralized peptic digest was centrifuged

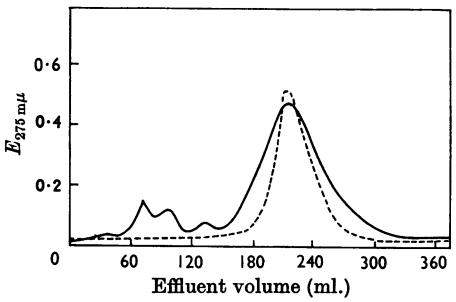

Fig. 1. Elution patterns of a peptic digest of catalase from Sephadex G-75 columns (2cm.×65cm.). The eluent was 0·1M-phosphate buffer, pH7·8. ——, Fraction B; – – – –, PAPDP.

at 0° to remove insoluble materials, and solid $(NH_4)_2SO_4$ was added, with constant stirring, to give 40% saturation. The precipitate that formed after standing for 1hr. at 0° was recovered by centrifugation. Two further precipitates were similarly obtained by raising the $(NH_4)_2SO_4$ concentration to 80% and then to 100% saturation.

The three precipitates [0–40%, 40–80% and 80–100% $(NH_4)_2SO_4$ saturation] are designated fractions A, B and C respectively. They were dialysed against ice-cold distilled water until free from $(NH_4)_2SO_4$, freeze-dried and stored at 0°.

(*c*) Further purification of fraction B. Fraction B was dissolved in 5ml. of 0·1M-phosphate buffer, pH7·8, and loaded on a column (2cm.×65cm.) of Sephadex G-75 (medium grade; Pharmacia, Uppsala, Sweden) that had been equilibrated with 0·1M-phosphate buffer, pH7·8. The column was developed at 0–5° with the same buffer, 5ml. of the effluent was collected and the extinctions of the fractions were measured at 275mμ. The elution pattern of the sample (Fig. 1) indicated one major component and a number of minor components.

The fractions containing the major component were pooled and solid $(NH_4)_2SO_4$ was added, with stirring, to give 80% saturation. The dark-brown precipitate that formed on standing for 1hr. was separated by centrifugation and the supernatant was discarded. The precipitate was dissolved in 600ml. of 0·015N-NH_3, and solid $(NH_4)_2SO_4$ was added with stirring to give 60% saturation. On adding 40ml. of 20% (w/v) trichloroacetic acid a dark-brown material was precipitated, which was recovered by centrifugation. This precipitate was dissolved in 5ml. of 0·1M-phosphate buffer, pH7·8, dialysed against the same buffer and subjected to chromatography on Sephadex G-75 column as described above. The brownish-yellow fractions were pooled, dialysed thoroughly against ice-cold distilled water and freeze-dried. This sample is designated PAPDP.*

Ultracentrifugal methods. Sedimentation was carried out in a Spinco model E analytical ultracentrifuge equipped with a phase plate and a constant-temperature control system. Experiments were carried out at maximum speed (59780rev./min.) in the analytical rotor AnD. The Spinco synthetic-boundary cell of the valve type described by Pickels, Harrington & Schachman (1952), with a 4° sector and a centre piece of 12mm. thickness, was used together with a counterbalance with the usual reference holes to provide reference points for determining radial distances of the photographs. The movement of the boundary was recorded by schlieren photographs at 16min. intervals from the time the rotor reached 66% of full speed.

Solutions of PAPDP were clarified at 10000*g* in the International model PR–2 refrigerated centrifuge to remove any suspended material. Sedimentation runs were performed at three different concentrations: 0·76, 0·5 and 0·3% (w/v) PAPDP in 0·1M-phosphate buffer, pH7·8. Patterns were photographed with yellow rapid Agfa spectral plates and a yellow filter. The deep-brownish colour of the PAPDP solutions rendered it difficult to record both sides of the sedimenting boundary satisfactorily. The use of red filters did not improve the patterns. The special red-sensitive plates recommended by Schachman (1957) for coloured materials such as haemoglobin were not available. However, the peaks were clearly visible after removing the ground-glass viewing screen and installing the optical viewer.

The PAPDP sedimented as a single symmetrical boundary. The sedimentation coefficients at different concentrations were calculated as described by Schachman (1957), by using a Hilger model H non-recording photoelectric microphotometer with a galvoscale projector to determine the position of the peak maximum. This region was clearly visible in the photographs.

Determination of molecular weight of PAPDP. The determination was carried out by the 'Archibald method' as described by Klainer & Kegeles (1955). Runs were carried out with the synthetic-boundary cell and the analytical rotor AnD. A solution containing only 3·2mg. of PAPDP/ml. was used since at higher concentrations the plates were difficult to read. A 0·6ml. sample of PAPDP solution was taken in the sector with the cup empty. The run was carried out at 23° and a speed of 14290rev./min., and photographs were taken at 16, 24 and 32min. after reaching 66% of maximum speed and at a phase-plate angle of 70°. Changes at the meniscus alone were recorded and not those at the cell bottom.

A second run was performed with 0·4ml. of the same PAPDP solution in the sector and 0·2ml. of the solvent in the cup. After the cup had emptied completely at a speed of about 9000rev./min. the rotor was accelerated slowly to a speed of 14290rev./min., and three photographs of the boundary at the higher speed and with the phase plate at the same angle of 70° were taken within 5–10min. The necessary measurements of the ultracentrifuge patterns were made at 10μ intervals with a two-dimensional Hilger L–50 measuring micrometer (Klainer & Kegeles, 1955). The deviations of the base line at and immediately after the meniscus position were read from the smooth curve obtained from the latter points on the graph, as at this region direct observations were not easy to make. Calculations were made as described by Schachman (1957).

Amino acid analyses. These were carried out with the standard automatic recording equipment described by Spackman, Stein & Moore (1958), with the accelerated method (Stark & Smyth, 1963). A dry sample (7mg.) of crystalline ox-liver catalase was hydrolysed with 6N-HCl for 20hr. at 110° in a sealed tube filled with N_2. The HCl

* Abbreviation: PAPDP, peroxidatically active pepsin-degraded product.

was then evaporated in a vacuum desiccator and the residue was dissolved in citrate buffer, pH 2·2 (Spackman *et al.* 1958). Some black particles were centrifuged down and the supernatant fluid, along with the washings of the sediment, was made up to 7·0 ml. A 5 mg. sample of PAPDP was hydrolysed in the same way.

RESULTS

Influence of pepsin on catalase. The catalatic activity of catalase was decreased on digestion with pepsin, whereas the peroxidatic activity was enhanced (Tables 1 and 2). The effect of pepsin on the peroxidatic activity of catalase is very similar to the effect of α-chymotrypsin (Radhakrishnan *et al.* 1963).

Peroxidatic activity of the fractions obtained after peptic digestion of catalase. The three fractions A, B and C exhibited peroxidatic activity, fraction B being the most active (Table 3). They were completely devoid of catalatic activity. Of all the fractions obtained from the peptic digest, PAPDP exhibited the highest peroxidatic activity. The peroxidatic activities shown by catalase and PAPDP are enzymic, since the boiled samples were devoid of peroxidatic activity.

Table 1. *Effect of pepsin on ox-liver catalase*

Experimental details are given in the text.

Digestion period (hr.)	Acid-insoluble N (% of total N)	Catalatic activity (% of control)
0	100	100
1	61	89
2	49	74
3	38	61
5	27	57
7	19	43
8	16	39
24	7	14

Table 2. *Influence of pepsin on the peroxidatic activity of ox-liver catalase*

Experimental details are given in the text. Peroxidatic activity was calculated as mg. of purpurogallin formed/mg. of protein nitrogen during 5 min. at 25° in the presence of H_2O_2 and pyrogallol.

Digestion period (hr.)	Peroxidatic activity	
	Test	Control
0	5·4	5·4
3	9·6	5·4
5	9·7	5·4
8	10·7	5·4
24	10·8	5·4

Sedimentation coefficient of PAPDP. For a 0·76% (w/v) solution of PAPDP the plot of $\log x$ (x being the distance of boundary to axis of rotation) against t was a straight line. The slope of this line, $d(\log x)/dt$, was substituted in the Svedberg equation:

$$S_{obs.} = \frac{1}{\omega^2} \cdot \frac{d(\log x)}{dt}$$

The observed sedimentation coefficients at different PAPDP concentrations were converted into $S^0_{20,w}$ (Schachman, 1957) and the value of the sedimentation coefficient at infinite dilution, 2·6 s, was obtained by extrapolation (Fig. 2).

Molecular weight of PAPDP. From the measurement of the changes at the meniscus recorded at three different time-intervals the molecular weight of PAPDP was calculated as 27 478 ± 664 (mean ± S.D.). The partial specific volume of PAPDP

Table 3. *Peroxidatic activity of the ammonium sulphate fractions of peptic digests of ox-liver catalase*

Experimental details are given in the text. Peroxidatic activity is expressed in the same units as in Table 2.

Fraction	Peroxidatic activity
Catalase	5·5
Boiled catalase	Nil
Neutralized peptic digest of catalase	10·8
0–40% satd. $(NH_4)_2SO_4$ (fraction A)	2·9
40–80% satd. $(NH_4)_2SO_4$ (fraction B)	7·1
80–100% satd. $(NH_4)_2SO_4$ (fraction C)	Nil
PAPDP	8·2
Boiled PAPDP	Nil

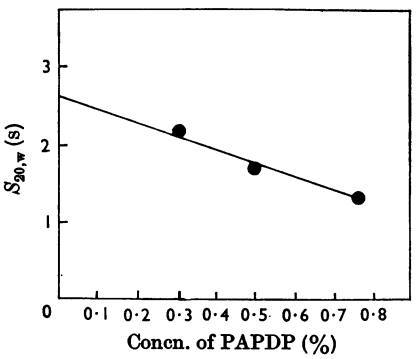

Fig. 2. Variation of $S_{20,w}$ with concentration of PAPDP. Sedimentation runs were carried out at 22–23° in 0·1 M-phosphate buffer, pH 7·8.

Table 4. *Some physical and chemical properties of PAPDP and catalase*

Experimental details are given in the text.

	Nitrogen (%)	Iron (%)	$S^0_{20,w}$ (s)	Mol.wt.	No. of amino acid residues/mol.
PAPDP	15·3	0·21*	2·6	27500	248
Catalase	16·2	0·09	11·4†	248000‡	2060

* Corresponds to minimum molecular weight of 26600.
† Samejima & Yang (1963).
‡ Sumner & Gralen (1938).

Table 5. *Comparison of the amino acid compositions of catalase and PAPDP isolated from the peptic digest of catalase*

Experimental details are given in the text.

	Amino acid composition (residues/mol.)				
	Catalase				Ratio of number of residues in catalase to number in PAPDP
Amino acid	Schnuchel (1956)	Schroeder *et al.* (1962)	Present study	PAPDP	
Lys	169	116	110	11	10·0
His	124	89	79	6	13·1
NH_3	—	—	271	42	6·4
Arg	114	130	119	9	13·2
Asp	214	294	281	29	9·6
Thr	70	96	102	13	7·8
Ser	75	97	104	18	5·7
Glu	165	197	189	27	7·0
Pro	99	166	174	23	7·5
Gly	109	155	151	25	6·0
Ala	120	154	158	16	9·8
CyS (half)	22	29	10 28*	16	1·7
Val	131	141	113	16	7·0
Met	48	41	40	6	6·6
Ile	73	79	61	10	6·1
Leu	165	154	146	12	12·1
Tyr	94	86	83	5	16·5
Phe	115	132	122	6	20·3

* Determined by the phosphotungstic acid method of Kassell & Brand (1938).

was assumed to be 0·725ml./g., since for most proteins it is in the range 0·70–0·75ml./g. (McMeekin & Marshall, 1952). The accuracy of the procedure was checked by measurements on crystalline bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex). A value of 68600 was obtained, which is in good agreement with the value of 68200 reported by Harrington, Johnson & Ottewil (1956). Further, the minimum molecular weight of PAPDP calculated from the iron content, namely 0·21% (Table 4), agrees closely with the value obtained by ultracentrifugal analysis and indicates the presence of a single haem prosthetic group in the molecule.

Paper electrophoresis of catalase and PAPDP. On paper electrophoresis in 0·05M-veronal buffer, pH 8·6, catalase migrated towards the anode, whereas PAPDP remained at the origin.

Amino acid composition of catalase and PAPDP. The amino acid compositions are summarized in Table 5. The results obtained by Schnuchel (1956) and Schroeder, Saha, Fenninger & Cua (1962) for ox-liver catalase are also included. Our results are, on the whole, nearer to the preliminary data of

Schroeder *et al.* (1962) than to the results obtained by Schnuchel (1956) by the paper-chromatographic technique.

DISCUSSION

The PAPDP isolated from the peptic digest of crystalline catalase in the present experiments has a molecular weight of 27500 and exhibits more peroxidatic activity than catalase. The fractions A, B, C and PAPDP obtained from the peptic digest of catalase together have lower peroxidatic activity than the digest itself. This can be attributed, at least in part, to losses in activity during the isolation of the fractions. The pepsin-modified catalase obtained by Anan (1958*b*) had a much higher molecular weight and exhibited fairly strong activities in the peroxidatic oxidation of guaiacol, the 'Nadi' reaction and the aerobic oxidation of L-ascorbic acid. These catalytic activities were strongly inhibited by cyanide, azide and fluoride.

Inada, Kurozumi & Shibata (1961) reported the dissociation of catalase into one-third size sub-units (molecular weight about 80000) in alkaline solutions and they attribute the generation of peroxidatic activity to this dissociation. Caravaca & May (1964) reported the isolation of an 'active peroxidase' from liver catalase after treatment with alkali. They found that their 'hepatocatalase-peroxidase' shared many properties in common with the well-characterized horse-radish peroxidase (Akazawa & Conn, 1958; Klebanoff, 1959). The peroxidatic activities of pepsin-modified catalase (Anan, 1958*b*), 'hepatocatalase-peroxidase' (Caravaca & May, 1964) and of PAPDP are, however, low and are not comparable with that of horse-radish peroxidase. Further, free protohaemin is devoid of peroxidatic activity (Tu, 1964). Samejima & Yang (1963) demonstrated that catalase dissociates in an acid medium into physically indistinguishable sub-units of molecular weight 120000, which are devoid of catalatic activity. The observation that incubation of catalase at pH 4·0 without pepsin does not result in an increase in peroxidatic activity (Table 2) suggests that denaturation alone is not responsible for the observed peroxidatic activity of the peptic digest. It is more likely that treatment with pepsin causes a breakdown to smaller active fragments. This conclusion is further supported by the finding that boiled PAPDP solution has no peroxidase activity. Similar studies have been made with cytochrome *c*. Thus Tsou (1951) obtained a pepsin-modified cytochrome *c* that was inactive in both succinate-oxidase and cytochrome-oxidase systems, but it was autoxidizable and exhibited ascorbate-oxidase activity and catalatic activity. Tuppy & Paléus (1955) purified the pepsin-modified cytochrome *c* obtained by Tsou (1951) and isolated a haemopeptide with peroxidatic activity.

The haemoproteins myoglobin, haemoglobin, peroxidase and catalase have protohaematin as their prosthetic group, but the attachment of different apoproteins gives them distinct and characteristic properties. These haemoproteins, in addition to their respective biological activities, also exhibit both catalatic and peroxidatic activities (Willstätter & Pollinger, 1923; Haurowitz, 1931; Keilin & Hartree, 1955). The peroxidatic activity of catalase was first demonstrated by Keilin & Hartree (1945), who suggested that this, rather than the ability to decompose hydrogen peroxide, might be the primary physiological function of this enzyme. The fact that a decline in catalatic activity accompanies an increase in peroxidatic activity could be due to the removal of certain fragments that are essential for catalase activity. However, it is also possible that the loss of such fragments may even alter the tertiary structure, exposing the active sites responsible for peroxidase activity.

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