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The Isolation and Kinetics of Lactoperoxidase

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The catalatic and peroxidatic activities of lactoperoxidase and horse-radish peroxidase are described. For measuring the peroxidatic activity. pyrogallol was used as a hydrogen donor. A manometric method was used for the determination of the Km value. The Km value for the catalatic reaction was calculated on the basis of O_2 evolved, and the peroxidatic reaction was calculated on the basis of CO_2 evolved. In the catalatic reaction the efficiency of lactoperoxidase and horse-radish peroxidase are of the same order. The peroxidatic activity of lactoperoxidase is higher than that of horse-radish peroxidase. The mechanism of peroxidase action was interpreted as described by Chance[2].

Peroxidase and catalase are regarded as enzymes with very similar properties and functions. The close resemblance between catalase and peroxidase can be seen in their reaction with hydrogen donors. The reaction of peroxidase with H_2O_2 and hydrogen donors can be schematically represented as follows

$$\text{Pse} \xrightarrow[k_1]{+H_2O_2} \text{Comp I} \xrightarrow[k_7]{+\text{ donor}} \text{Comp II} \xrightarrow[k_4]{+\text{ donor}} \text{Pse} + \text{oxidized donor}$$

Compound I of catalase can be reduced to Compound II from which catalase like peroxidase, can be liberated by exogenous donors[1]. Compound I of catalase and peroxidase have similar absorption spectra and show similar kinetic behaviour[2].

The object of our study was to determine peroxidase activity of lactoperoxidase and horse-radish peroxidase in the presence and absence of added donors.

For the purpose of the present study a method of obtaining the enzyme lactoperoxidase was required. A suitable simple method was developed, and is briefly described in this paper.

EXPERIMENTAL

Materials and Methods

Horse-radish peroxidase used in our experiments was purchased from C.F. Boehringer & Soehne GmbH, Mannheim, with RZ ca. 0.3[3].

The isolation of LP was carried out according to Morrisson *et al.*[4] with modifications.

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* Abbreviations: Pse, peroxidase; LP, lactoperoxidase; HRP, horse-radish peroxidase.

From fresh, unpasteurized skim milk (3 liter) containing 2.95% protein, the yellow green whey solution was separated after the action of 150 mg. rennet. The whey was adjusted to *p*H 7.0, and treated with 10 g/l of the ammonium form of the resin IRC-50. The mixture was stirred at room temperature until the remaining liquid did not give positive benzidine test.

The resin was removed by filtration, washed with distilled water and transferred into a glass column (60×1 cm. diameter). The elution of the enzyme from the resin was accomplished by adding successively 75 ml. of cold 1.0 *M* K_2HPO_4 at 3°C. The first 30 ml. of the eluate were discarded and the next 30 ml were collected and dialyzed against 0.01 *M* phosphate buffer (*p*H 5.9) for 48 hours at 3°C, with four changes of the buffer solution. The resulting enzyme preparation containing 0.347 mg. protein wt/ml, was used for paper electrophoresis and for the estimation of peroxidase activity.

The rest of 1.0 *M* K_2HPO_4 solution was allowed to stand in the column overnight at 3°C and 25 ml. of eluate was obtained giving a positive benzidine test. This enzyme preparation was dialyzed in the same manner as described above and used also for the estimation of peroxidase activity.

In another experiment the eluate was electrodialyzed for about 20 minutes against 0.01 *M* phosphate buffer (*p*H 5.9), starting with a current of about 20 mA and 95 V, and ending with 80 mA and 45 V.

The gel filtration technique with Sephadex G-25 was used for the concentration of the eluate from IRC-50 column. The eluate was mixed with dry Sephadex G-25, the slurry put in a column (15×0.8 cm. diameter) and eluted with 0.01 *M* phosphate buffer *p*H 5.9. In the first 5 ml of the eluate maximum concentration of LP was found and the ratio 412 mμ / 280 mμ was 0.47. In total 20 ml of the eluate were collected.

Nitrogen was determined by the Kjeldahl method using the distillation apparatus of Markham[5], and the mixed bromcresol green + methylol red indicator of Ma & Zuazaga[6].

The absorption spectra were recorded on a Perkin-Elmer spectrophotometer with a quarz monochromator and on a Zeiss spectrophotometer.

Electrophoresis was carried out in a Durrum-type paper electrophoresis apparatus. Electrophoresis experiments were carried out in the conventional manner, in veronal buffer *p*H 8.6. During the runs a constant current of 8V/cm, and 0.3—1 mA/cm. was allowed to pass for four hours. At the end of the run the paper strips were placed in the oven at 110°C for 30 minutes, and then stained with 0.3% alcoholic solution of ninhydrin. The elution of the obtained protein bands from the unstained paper strip was carried out with a 10% solution of NaCl during 24 hours.

For estimation of peroxidase activity Warburg microdifferential manometers were used at 35°C for LP, and at 20°C for HRP, with air as the gas phase.

RESULTS

The LP preparation obtained after dialyzing the eluate from the IRC-50 column against 0.01 *M* phosphate buffer contained 0.347 mg. protein wt/ml. determined by the Kjeldahl method. The optical density at 280 mμ (1 cm cell) was 0.232 and multiplied by the factor 1.43 which has been experimentally determined by Morell[7] gave the protein concentration of 0.332 mg/ml. This is in accordance with the result obtained by the Kjeldahl method.

The purity of the various LP preparations was determined by measurement of the haem absorption (Soret band 412 mμ) relative to that of the protein (280 mμ). In this method the percentage of purity of the enzyme is defined by the expression: 1.11 × optical density at 412 mμ × 100 / optical density at 280 mμ, where 1.11 is the ratio of the optical density at 280 mμ, to that of 412 mμ calculated for the pure enzyme[8].

The purity of the eluate received directly from the IRC-50 column was 45%, and after concentration with Sephadex G-25 a purity of 47% was obtained. The electrodialyzed preparation had a purity of 95.5%, but the enzyme was inactive.

The electropherogram of the enzyme preparation from the winter milk shows four proteins, one of which moved towards the cathode. Measuring the absorption spectra of the various bands, we found, that the peroxidase fraction moving towards the anode was LP B, and that LP A was moving towards the cathode. The remaining two bands were proteins showing no absorption at 412 mμ. This is in accordance with the earlier findings[4].

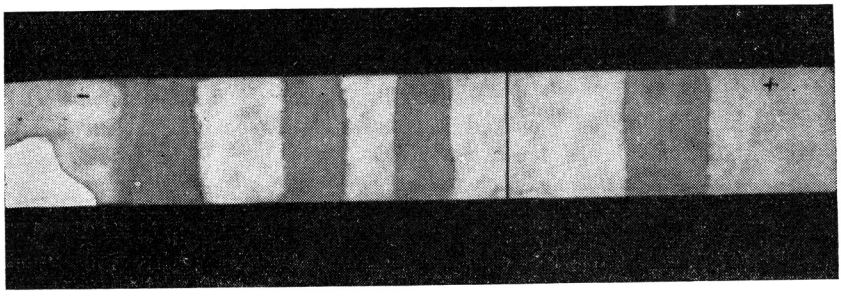


Fig. 1. Separation of lactoperoxidase by paper electrophoresis. Veronal buffer, *p*H 8.6, $\mu = 0.05$, 8.0 V/cm for 4 hours. Whatmann N° 1 filter paper, 3×32.5 cm. Durrum type apparatus. From left: protein fraction, LP B, red protein and LP A.

Effect of Increasing Concentration of Lactoperoxidase on Velocity of the Catalatic Reaction

The experiments were carried out at 35°C in Warburg manometers. The main part of the flask received 1, 1.5, 2, 2.5 ml of the LP preparation,

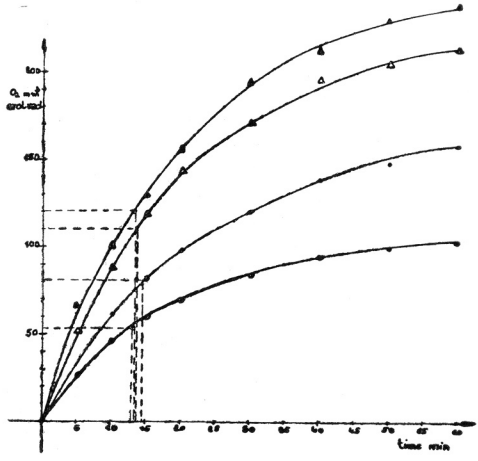


Fig. 2. Effect of different concentration of lactoperoxidase on velocity of the catalatic reaction. Each manometer flask contained: a constant concentration of $3.15 \times 10^{-2} M$ H_2O_2 in *M*/15 phosphate buffer *p*H 5.9, and Preparation as indicated; total vol. 2.8 ml. Added LP preparation in ml.:

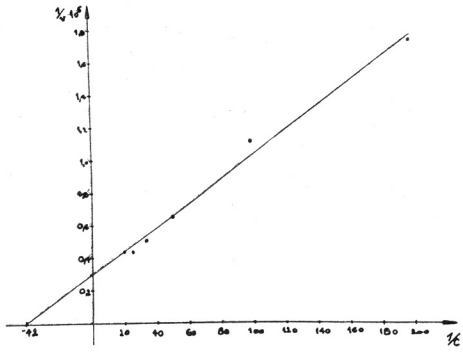
○, 1 ml; ● 1.5 ml.; ∠, 2 ml.; ▲, 2.5 ml.

obtained by dialyzing against 0.01 *M* phosphate buffer (*p*H 5.9), the eluate from the IRC-50 column. The side bulbs received 0.3 ml. 1% H_2O_2 in *M*/15 phosphate buffer, *p*H 5.9. Each flask received water when necessary to obtain a total volume of 2.8 ml. The O_2 output was recorded for 70 min. at 5 min. intervals. Fig. 2. shows that the reaction velocity was maximum in the first 5 minutes, and the amount of evolved O_2 was proportional to the quantity of enzyme used. Half of the total evolved O_2 was obtained after 13.5 mins. regardless of the quantity of LP used in the experiment.

The Manometric Method for Estimation of Peroxidase Activity

A) *Determination of Michaelis Constant (Km) of Lactoperoxidase for the Catalatic and Peroxidatic Reactions*

To determine the Michaelis constant (Km), increasing concentration from 0.5×10^{-2} to 5×10^{-2} *M* H_2O_2 in *M*/15 phosphate buffer *p*H 5.9 was placed in the flasks, and the side bulbs received a constant volume (0.5 ml) of LP preparation (the dialyzed eluate from the IRC-50 column). The total volume in each flask was 2.5 ml. The Km value for enzyme — hydrogen peroxide reaction found was 2.38×10^{-2} *M*. The Km value was calculated from the rate of O_2 evolved (Fig. 3).



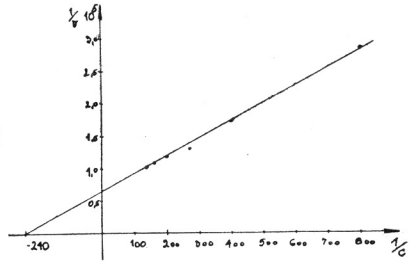


Fig. 3. Graphic evaluation of Michaelis constant of lactoperoxidase for the catalatic reaction, using an enzyme preparation of 45% purity, c = m*M* conc. of H_2O_2, v = velocity in activity test. Results plotted according to method of Lineweaver & Burk[9].

Fig. 4. Graphic evaluation of Michaelis constant of lactoperoxidase for the peroxidatic reaction, using an enzyme preparation of 45% purity, with pyrogallol as a donor. c = m*M* conc. of H_2O_2, v = velocity in activity test. Results plotted according to method of Lineweaver & Burk[9].

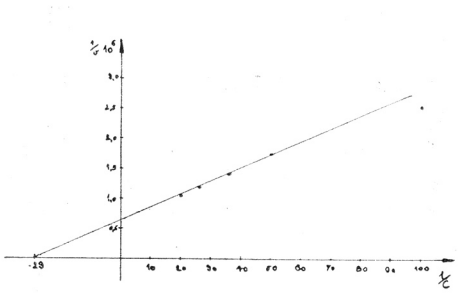
The experiments were carried out with added pyrogallol as a donor in a final concentration of 2.5×10^{-2} *M*[10]. The increasing concentration from 1.25×10^{-3} to 7.5×10^{-3} *M* H_2O_2 in *M*/15 phosphate buffer *p*H 5.9 was placed in the main flasks, and the side bulbs received a constant volume (0.5 ml.) of LP preparation.

We assume, that during the experiment catalatic and peroxidatic reaction proceeded and CO_2 and O_2 evolved. The value for Km was calculated from the difference between the rate of evolution of CO_2 and O_2 (Fig. 4).

The average value for Km found was 4.15×10^{-3} *M*, in good agreement with the approximate value calculated by Chance[11].

B) *Determination of Michaelis Constant (Km) of Horse-Radish Peroxidase for the Catalatic and Peroxidatic Reactions*

Experiments were carried out in Warburg manometers at 20°. The first set of experiments was carried out without added donor. In the main part of the flask the increasing concentration from 1×10^{-2} to 4×10^{-2} *M* H_2O_2 in *M*/15 phosphate buffer was placed. The side bulbs received a constant volume (0.5 ml) of HRP (Boehringer & Soehne) in a concentration of 3.2 mg/ml in the same buffer. The total volume in each flask was 2.0 ml. The Km value was calculated from the rate of O_2 evolved and was found to be 3.45×10^{-2} *M* (Fig. 5).



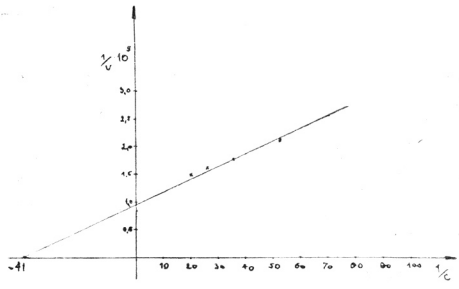


Fig. 5. Graphic evaluation of Michaelis constant of horse-radish peroxidase for the catalatic reaction, using an enzyme preparation purchased from Boehringer & Soehne. c = m*M* conc. of H_2O_2 v = velocity in activity test. Results plotted according to method of Lineweaver & Burk[9].

Fig. 6. Graphic evaluation of Michaelis constant of horse-radish peroxidase for the peroxidatic reaction, using an enzyme preparation purchased from Boehringer & Soehne. c = m*M* conc. of H_2O_2, v = velocity in activity test. Results plotted according to method of Lineaweaver & Burk[9].

In the second set of experiments the same quantity of HRP (Boehringer & Soehne) and hydrogen peroxide were used, but with added pyrogallol as a donor in a final concentration of 2.5×10^{-2} *M*. The experiments were carried out in the same manner as above. The Km value was calculated from the rate of the gases evolved, as it was described for the LP peroxidatic reaction. The Km value was found to be 2.44×10^{-2} *M* (Fig. 6.).

DISCUSSION

Peroxidatic oxidation of H_2O_2 *i.e.* its catalatic decomposition of added H_2O_2 to O_2 and water is a property common to all haemoproteins.

The main object of the present study was to asses the relative catalatic activities of lactoperoxidase and horse-radish peroxidase. The catalatic reaction *i.e.* disappearance of substrate (H_2O_2) was determined manometrically. Since in our experiments the concentration of the enzyme, as well as other experimental conditions were fixed, the decomposition of H_2O_2 could be followed under comparable conditions.

Lactoperoxidase was found able to catalyse the oxidation of H_2O_2 and the Km value was found to be 2.38×10^{-2} *M*. The peroxidatic activity of lactoperoxidase, using pyrogallol as the donor, was determined and the Km value found was 4.15×10^{-3} *M*. According to Chance[2] compound II is the rate limiting intermediate.

Morell[7] has calculated the value of k_8 from the equation

$$Km = \frac{k_8 \cdot a}{k_1 (1 + k_8/k_4)} \qquad (1)$$

by putting $k_7 = k_8 \cdot a$.

If we take an approximation to equation (1)

$$Km = \frac{k_4 \cdot a}{k_1} \qquad (2)$$

we obtain $k_4 = 3.65 \times 10^6\ M^{-1}\ sec^{-1}$, assuming $k_1 = 2.2 \times 10^7\ M^{-1}\ sec^{-1}$ and the values of Km and *a* given in the text.

The apparent peroxidatic activity of horse-radish peroxidase observed is relatively far too low in comparison with the peroxidatic reaction of lactoperoxidase. This suggests that(1) the enzyme was for some reason highly inhibited or(2) catalase was present in the preparation[12].

It is difficult to suppose that the horse-radish peroxidase from C.F. Boehringer & Soehne would contain a high percentage of catalase. The low values obtained are perhaps due to a high concentration of H_2O_2 for Keilin and Hartree (loc. cit.) worked with a concentration of H_2O_2 which was approximately a hundred times lower.

The catalatic reaction of horse-radish peroxidase with lower H_2O_2 concentration will be the object of a further study.

The experimentally determined Km values for the catalatic and peroxidatic reactions of horse-radish peroxidase were 3.45×10^{-2} *M* and 2.44×10^{-2} *M*, respectively.

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IZVOD

Izolacija i kinetika laktoperoksidaze

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Michaelisova konstanta Km laktoperoksidaze određena je Warburgovim manometrom. Mjerenja su vršena uz dodatak pirogalola kao donora vodika, a vrijednost Km je izračunata iz volumena nastalog CO_2. Michaelisova konstanta laktoperoksidaze određena je i bez dodatka donora, samo uz vodikov peroksid. Mjerenjem volumena razvijenoga kisika izračunata je brzina reakcije kod raznih koncentracija vodikova peroksida, te je iz ovih podataka nađena Michaelisova konstanta Km, koja iznosi 2.38×10^{-2} *M*. Iako je dobivena konstanta veća od Michaelisove konstante određene uz dodatak pirogalola (4.15×10^{-3} *M*) nesumnjivo postoji jasno izražena enzimatska aktivnost.

Određena je Michaelisova konstanta Km i za peroksidazu iz hrena, i to sa dodatkom i bez dodatka donora. Pokazalo se, da je peroksidaza iz hrena reagirala s vodikovim peroksidom bez dodatka donora, a nađena Km vrijednost iznosi 3.45×10^{-2} *M*.

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