PREPARATION, PURIFICATION AND CRYSTALIZATION OF HORSE-RADISH PEROXIDASE COMPONENTS

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(Received July 28, 1967)

A simple method has been described for the preparation of pure horse-radish peroxidase. Electrophoretic studies of the peroxidase revealed the presence of five components. The four major components of the enzyme were separated in a purified form by ion exchange chromatography. Fine needle-shaped crystals were obtained under the conditions of 15 mg/ml enzyme and 55 % saturation of ammonium sulphate.

Introduction

Peroxidase is a very widely distributed enzyme among high plants. The enzyme has been prepared from such diverse sources as figs., [1] horse-radish, [2,3] yeast, [4] turnip, [7] and Japanese radish. [5,6] The enzyme has not been isolated from plants in a rather purified state except in the case of horse-radish. [15] Theorell [9,10] was the first to crystallize the enzyme and subsequent workers have used substantially the same method.

Its enzymatic, physical and chemical properties have been studied by different workers. [14,15] It has been established that preparation from horse-radish, sweet potatoes, japanese radish, turnip and other luguminous plants has multiple enzymatically active components. Theorell[10] reported two different peroxidases, referred to as I and II, from horse-radish. Subsequently, Keilin and Hartree[3] found that when pure peroxidase was kept at 0°C for some time it gave rise to a brown compound with somewhat deminished enzymatic activity. Later Jermin, [11] using paper electrophoresis, observed that peroxidase preparations from different kinds of plants have between two and five components.

The present report describes the preparation, purification, separation and crystallization of the different components of horse-radish peroxidase.

Experimental

Sources of Horse-radish Roots.—Horse-radish roots were gathered in August from an Indiana (U. S. A.) farm and also were brought by Hollmann's Commission Company, St. Louis, Mo., U. S. A.

Determination of the Peroxidase Activity.—The activity of the peroxidase preparation was estimated by the method of Summer and Gjissing[12] with slight modification. The P. Z. (purpurogallinzahal), i. e., mg. of purpurogallin formed by 1 ml. of enzyme preparation in 5 min from pyrogallol and H_2O_2 under fixed conditions) was determined by Willstatter and Stoll's method. [13]

Gel Electrophoresis Technique.—Gelman sepraphore III polyacetate cellulose strips were soaked in 0.1 M sodium acetate buffer (pH 5.0) for about 10 min for restoring their original gel structure. Then the sample was applied after blotting the strip between two absorbant blotting papers. The electrophoretic analysis was run in 0.1 M sodium acetate buffer (pH 5.0) for 1 hr at 250 volts in the cold room at 5°C. The strips were stained with buffalo black and then they were cleared in a mixture of acetic acid and ethanol.

Results

Preparation of the Enzyme Peroxidase

The method chosen in this work was a simple method which avoids the use of unavailable Tiselius electrophoresis used by Theorell, and reduces the number of fractionation steps used by other workers.

Step 1 : Isolation of Peroxidase from Horse-radish Roots.—10 kg washed roots were cut into small pieces and minced. The mince was extracted with 14 l. distilled water overnight in the cold room, and then squeezed by hand through muslin (P.Z. 1.1).

Step 2 : Treatment with Ethanol and Chloroform.—5 l. ethanol: chlorofrom (2:1 v/v) were left to cool at 0°C and added to the enzyme extract. The mixture was stirred for 10 min. The residue was centrifuged in a refrigerated centrifuge, and the clear yellow supernatent (21 l.) was concentrated by distillation in a rotary distilling apparatus under reduced pressure to about one-fourth of its volume. The temperature was kept at 30°C under reduced pressure so as not to denature the enzyme.

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Step 3: *Fractionational Precipitation with Ammonium Sulphate.*—To each litre of the concentrate 200 g ammonium sulphate was added and the inactive precipitate formed was centrifuged and discarded. Then another 200 g ammonium sulphate per litre of the original concentrate was added to the supernatant. The precipitate which contained all the activity was centrifuged in a refrigerated centrifuge, and then dissolved in 50 ml of distilled water, and dialysed against distilled water overnight.

Step 4 : *Fractional Precipitation with Ethanol.*—The enzyme solution was cooled to 0°C and cold ethanol (0°C) was added (1.5 vol). The precipitate was centrifuged in a refrigerated centrifuge, then dried *in vacua* and its activity was tested (P. Z. 50). To the supernatant, ethanol (2.3 vol) was added. After standing at 0°C for 30 min the suspension was centrifuged. The precipitate was dried *in vacuo* and the enzyme activity was tested (P. Z. 600). The precipitate was suspended in 50 ml distilled water and again fractionally precipitated with ethanol under the same conditions as mentioned above except that the fractions were collected between 0 and 1.3 vol (fraction A) and between 1.3 and 2.2 vol (fraction B). These fractions were dried *in vacuo* (activity of fraction A, P. Z. 100; and fraction B, P. Z. 900). Fraction B was dissolved in 20 ml H_2O and again fractionated with ethanol. The precipitate with 1.5 vol (fraction C) and between 1.5 and 2 vol ethanol (fraction D) were collected, dried *in vacuo* and the activity tested, (fraction C, P. Z. 410; fraction D, P. Z. 1000); Fraction D weighed about 150 mg.

Existence of Multiple Components of Horse-radish Peroxidase

Gel electrophoresis was used to test the purity of fraction D. Figure 1 shows the electrophoretic pattern. Component 1 moved towards the anode and the rest of the other components 2, 3, 4 and 5 moved towards the cathode.

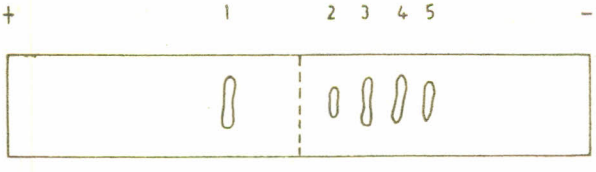

Fig. 1.—Sepraphore III gel electrophoretic pattern of purified horse-radish perexidase.

Separation of the Mutiple Components of Horse-radish Peroxidase

The components of horse-radish peroxidase preparation were separated by using ion exchange column chromatography. Carboxymethyl cellulose (Carl Schleicher & Schuell) powder (standard grade) was used. The powder was equilibrated with buffer sodium acetate pH 4.6 (30 ml 1M acetic acid and 15 ml 1M NaOH). The column was filled with buffer, and then carboxymethyl cellulose was introduced and allowed to settle without pressure until no further decrease in volume was noticed. Then the column was washed for some hours with cold distilled water in the cold room. The horseradish peroxidase was dialysed against H_2O overnight.

The enzyme sample was applied on the column, and by using the fraction collector H_2O was used as eluting material. A distinct brown band A goes down rapidly with H_2O and the rest of the material on the top of the column does not move at all. When the eluted material became colorless, the coloumn was treated with 0.1M acetate buffer (pH 4.2). At this lower pH, component C and component D moved hardly at all, but component B came down as a distinct sharp band.

The pH of the acetate buffer was changed to 4.6 components C and D began to separate and moved down. Component C moved much faster than D. When C was down all the way, the pH of the buffer was changed to pH 4.9 to speed up the mobility of D.

Only four fractions were separated by using the fraction collector while the fifth protein located on the electrophoretic pattern was too small to be detected on the column. The portein content of the different components in the eluted material was examined by using a Beckman spectrophotometer, and the protein absorbance at 280 mμ was measured (Fig. 2).

Concentration of Fractions.—The effluent fractions were concentrated by precipitation with 70% statured ammonium sulphate and the precipitates were redissolved in small quantities of distilled water and dialysed against water overnight.

Electrophoresis of Isolated Components.—For identifying the separated components, electrophoresis was conducted on each separate component and a comparison was made between each of these strips and the strip of the original enzyme preparation before fractionationn (Fig. 3).

Crystallization of Horse-Radish Peroxidase

The main purpose of this work was to prepare

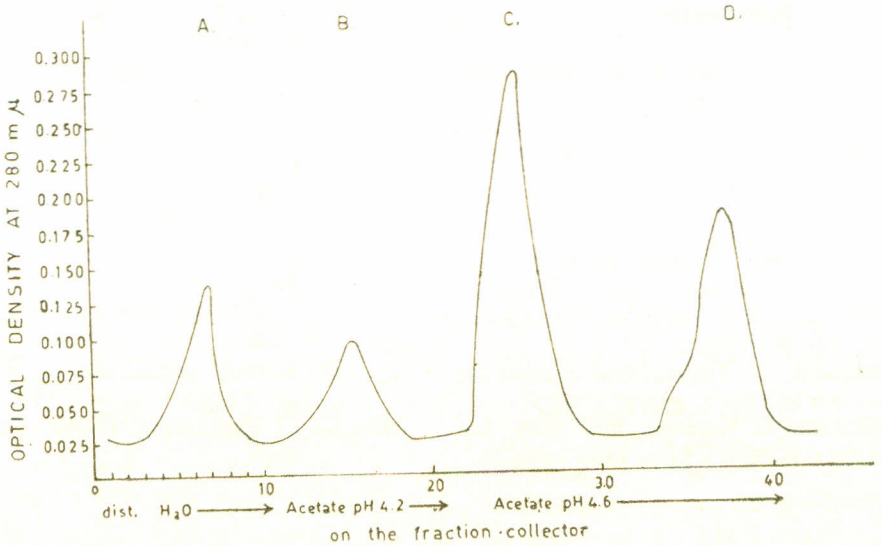

Fig. 2.—Chromatography of purified horse-radish peroxidase.

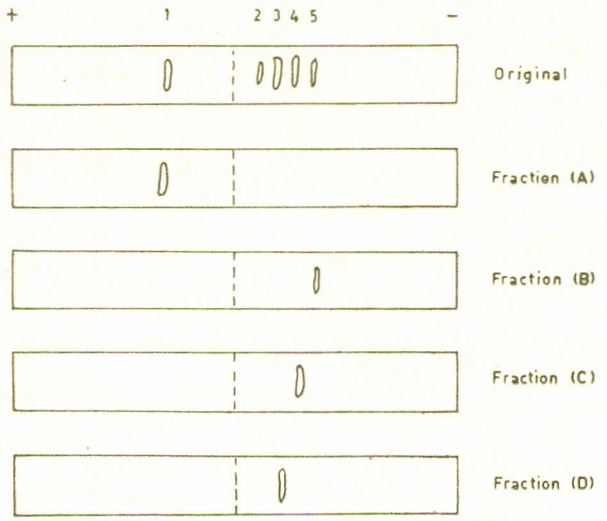

Fig. 3.—Diagram demonstrating the purity and location of pure components. Electrophoresis carried out on 0.1M acetate buffer pH 5.0.

a stable preparation of highly purified crystalline peroxidase. Attempts were made to crystallize the peroxidase from the isolated components. Different experiments were set up using the salting out technique by ammonium sulphate, using different concentrations of enzyme solutions with different concentrations of ammonium sulphate. A solution of 15 mg/ml horse-radish peroxidase at 55% saturation of ammonium sulphate showed the characteristic shimmer of a crystalline suspension after three weeks. Microscopical examination showed that the supsended material consisted of very fine needles.

Discussion

A simple method is described for the preparation of pure peroxidase from horse-radish roots. It avoids the use of Teselius electrophoresis and considerably reduces the number of preparation steps. The method depends on the removal of inactive material from the crude extract by precipitation with a mixture of chloroform and ethanol, and fractional precipitation with ammonium sulphate and ethanol.

The use of the electrophoresis technique on cellulose acetate strips revealed the presence of five different bands on the strips.

Four out of five components of the enzyme proteins were seperated. The fifth was too small to be detected. They were separated most conveniently in the pH range of 4.2 and 4.9.

An attempt was made to grow the crystals of these single band components of horse-radish peroxidase. Under the condition of 15 mg/ml and 55% ammonium sulphate saturation, some very fine crystals were obtained after 3 weeks.

Acknowledgement.—The author wishes to thank Dr. M. G. Rossmann, Biological Science Department, Purdue University, Indiana, U.S.A., for all the facilities offered during the course of this work.

This work was supported by a grant from the National Science Foundation, U. S. A.

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