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Isoelectric Fractionation, Analysis, and Characterization of Ampholytes in Natural pH Gradients

VII. The Isoelectric Spectra of Fungal Laccase *A* and *B*

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The possible heterogeneity of fungal laccase *A* and *B* from the fungus *Polyporus versicolor* has been investigated by the method of isoelectric focusing in natural, stable pH gradients.[1–4] Laccase *A* was resolved into two components, isoelectric at pH 3.07 and 3.27, and laccase *B* into ten components, isoelectric between pH 4.64 and 6.76. The specific activities of the two components in laccase *A* were approximately the same, whereas the components of laccase *B* showed marked difference with respect to activity. Both components of laccase *A* and most components of laccase *B* had strong blue colour and thus contain the spectrally unique Cu^{2+} ion previously described. Thus the data support the earlier conclusion that every enzyme molecule contains one atom of each of two different forms of Cu^{2+}.

Laccase (*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2.) from the fungus *Polyporus versicolor* has been found to exist in two chromatographically separable forms designated *A* and *B*.[5,6] Recently it was shown that both laccase *A* and *B* contain two atoms of Cu^{+} and two atoms of Cu^{2+}.[5,7] Further it was found that there are two types of Cu^{2+} according to electron paramagnetic resonance (EPR) spectra in both laccase *A* and *B*, and that there always is a 1:1 ratio of these two forms of Cu^{2+} in all preparations.[7] Based on these facts it was suggested that each enzyme molecule contains, together with two atoms of Cu^{+}, one atom of each form of Cu^{2+}. However, it could not be excluded that there were two kinds of enzyme molecules, each containing only one form of Cu^{2+}. If so, it might be possible to separate them with a sensitive and accurate separation method, especially if the chemical composition were different. If they had the same chemical composition, they might still have a difference in net charge depending on the difference in binding of the two types of Cu^{2+}.

This paper reports the results from isoelectric focusing experiments of the two chromatographic fractions *A* and *B* of fungal laccase. This method was chosen because it must be considered the most sensitive method available today for separation of protein molecules with small difference in net charge (for example, see Ref. 8). Although laccase *A* was resolved into two components and laccase *B* into ten components, it is shown that all components must contain both forms of Cu^{2+}.

MATERIALS AND METHODS

Proteins. Fungal laccase *A* and *B* were prepared by the method of Fåhraeus and Reinhammar.[5] The enzyme solutions were stored at −30°C in 0.1 M phosphate buffer, pH 6.0. They were dialysed against distilled water before the isoelectric focusing experiments.

Chemicals. The carrier ampholytes, available in the form of 8 % and 40 % water solutions and in different pH intervals (trade name Ampholine), were purchased from LKB-Produkter, Box 76, Stockholm-Bromma 1, Sweden. Sucrose, analytical reagent grade, from Mallinckrodt Chemical Works, St. Louis, Mo., USA, was used for the density gradient. All other chemicals used were of analytical grade.

Electrolysis apparatus. An electrolysis column of 110 ml volume from Ingenjörsfirman Consulta, Sköndalsvägen 106, Stockholm-Farsta, Sweden, was used.

Preparation of the column and isoelectric focusing by electrolysis. This was essentially done as described by Vesterberg and Svensson in No. IV of this series.[4] However, to prepare the density gradients we used a special device, working with a dense and a less dense solution and giving a constant density gradient. The device is to be described in the near future by H. Svensson and S. Pettersson.* The dense solution, with a density corresponding to 500 g sucrose/l, was prepared by mixing 9/10 of the carrier ampholyte solution with a stock solution, containing 900 g sucrose/l, the less dense by mixing 1/10 of the carrier ampholyte solution with distilled water. The concentration of carrier ampholytes was 0.5 % or 2 %, varying in the different experiments.

In some experiments the enzyme solution was mixed with the whole of the less dense solution. Then the enzyme will be found everywhere in the column from the start. In other experiments the pH gradient was first allowed to develop. After that, the enzyme was introduced in the form of a zone at a place in the column where the pH was harmless for the enzyme. Both these procedures gave the same results.

The final voltage at +4°C was about 700 V. In order to get the pI values at +25°C the electrolysis was, after the steady state was reached, continued for about 4 h with thermostated water of +25°C circulating through the cooling mantle. The voltage was then about 500 V.

pH and spectroscopic measurements. In order to measure pH and UV absorption (280 nm) an assembly according to Fig. 1 has been used (details will be published later). The effluent tube of the column is connected to a capillary tube leading to flow through cells for continuous measurements of pH and UV absorption. The pH measurements were made with a Beckman Expandomatic pH meter, equipped with a Beckman 97633 capillary pH electrode assembly and a Beckman ten-inch, linear, potentiometric recorder. The measurements as well as the calibration of the pH meter with standard buffer solutions (with an accuracy of ±0.02) were performed at +25°C, the same temperature as that of the final focusing of the column. For the UV absorption measurements a modified Vitatron (address Vitatron N.V. Dieren, Holland) universal photometer UFD, equipped with a flow through cell and a Vitatron linear/logarithmic integrating recorder was used. The recorders give the pH and UV courses of the column solution. The enzyme zones can be seen as sharp peaks on the UV absorption curve and their pI values can be easily derived from the pH curve. For subsequent enzyme activity measurements, fractions were collected manually. The size of the fractions were varied according to the distribu-

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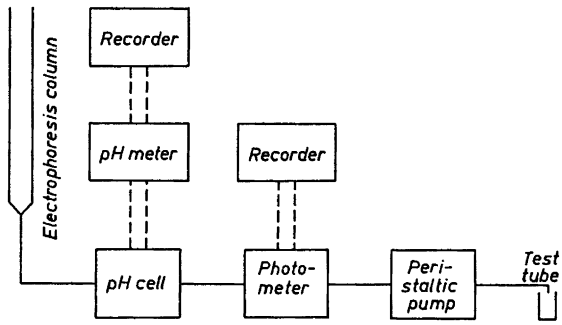

Fig. 1. A schematic diagram of the instrument set-up for the continuous measurement of pH and UV absorption courses of the column solution.

tion of the enzyme zones in the column. This could be done simply by following the UV absorption course. To secure good resolution in the regions where the enzymes were focused, these fractions were chosen as small as 0.7 ml. From other regions of the column larger fractions were taken.

Enzyme activity measurements. The enzyme activity was measured by a spectrophotometric method described earlier [9] with *N,N*-dimethyl-*p*-phenylenediamine as substrate with the exception that EDTA was excluded from the substrate medium. The photometer used was a Zeiss PMQ II coupled to a potentiometric recorder and the reaction was performed at 25°C.

The activity of the enzymes is expressed as the increase in absorbancy at 323 nm per minute per μl enzyme added to 3 ml substrate in a 1 cm cell. The enzyme samples were taken from the fractions obtained by emptying the isoelectric focusing column without further treatment, and they thus contained both sucrose and carrier ampholytes.

RESULTS

Laccase B. In a preliminary run on a gradient ranging from pH 3 to 10, the enzyme was separated into several components, isoelectric between approximately pH 4.5 and 6.8. Therefore, the following experiments were performed with carrier ampholytes isoelectric between pH 5 and 8, giving a shallow pH gradient in this interval. Due to the phosphoric acid at the anode, a satisfactory gradient is also obtained between pH 4.5 and 5. Fig. 2 shows the enzyme activity, UV absorption and pH as functions of the level in the column, in a representative run. 47.5 mg enzyme was used in this experiment. Nine fully resolved components can be seen. In another run one more peak has been obtained at pH 6.76. Even in the experiment described there is some activity at the basic side of the peak at pH 6.56.

For the basic protein cytochrome c, Flatmark and Vesterberg [10] have found a dependence of the pI values on the sugar concentration. To examine if such a dependence exists for laccase *B*, experiments were performed with the opposite current direction. The proteins will then be focused at other sugar concentrations in the column. No effect on the pI values was observed. This is in agreement with the result Vesterberg [8] has found for the components of myoglobin, isoelectric between 6.8 and 7.8.

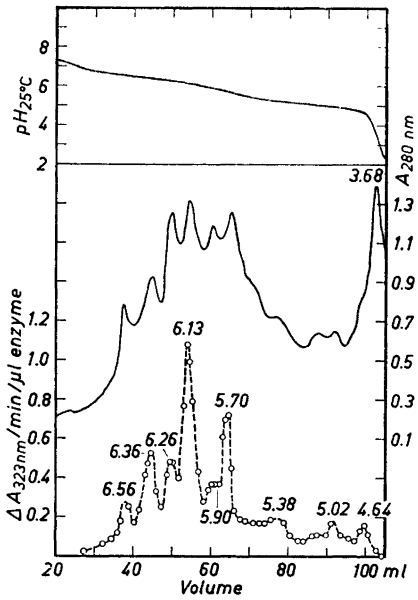

Fig. 2. pH (+25°C) course (upper solid curve), UV absorption (280 nm) course (lower solid curve), and enzyme activity course (O- - -O) of the column solution after isoelectric separation of fungal laccase *B*. The figures above the enzyme activity peaks give the pI values of the enzyme components. The UV peak at pH 3.68 is due to a low-molecular yellow compound supposedly pigment which is strongly bound to the enzyme. In the UV absorption curve there is a background absorbancy of about 0.20 which is due to the carrier ampholytes.

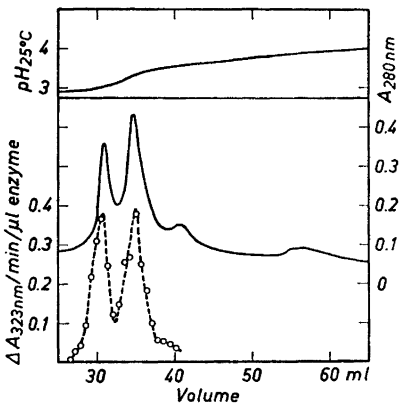

Fig. 3. pH (+25°C) course (upper solid curve), UV absorption (280 nm) course (lower solid curve), and enzyme activity course (O- - -O) of the column solution after isoelectric separation of fungal laccase *A*. The background UV absorbancy of about 0.08, which can be seen on the UV absorption curve, is due to the carrier ampholytes.

Laccase A. As with laccase *B* the first run was made on a pH gradient ranging from 3 to 10. The enzyme was focused in a steep portion of the gradient, at about pH 3.1. Such a portion of the gradient does not allow any good separation and there will be a great uncertainty in the pI measurements. Therefore, the following experiments were performed with carrier ampholytes, isoelectric between pH 3 and 5 to which has been added acetic acid to make the slope from pH 3 to the pH at the anode less steep. Then two components, isoelectric at pH 3.07 and 3.27 were found. 4.1 mg enzyme was used in the experiment shown in Fig. 3. As a complement, experiments were performed on gradients made without the Ampholine carrier ampholytes. Glutamic acid, aspartic acid, acetic acid, citric acid, phosphoric acid, and formic acid were used, giving a gradient between approximately pH 1 and 3.3 (details about these

acid gradients will be published later). Absolute agreement between experiments with these different types of gradients was obtained. The dependence of the pI values on the sugar concentration was examined in the same way as for laccase *B*. No effect on the pI values was observed.

DISCUSSION

The results from the isoelectric focusing experiments with laccase *A* showed that there are two components in this fraction and the amount of each component is about the same, if it is assumed that they have the same extinction at 280 nm (Fig. 3). This finding supports the suggestion that there might be two kinds of protein molecules in the ratio 1:1 each one containing only one form of Cu^{2+} as it has been found that there are equal amounts of the two forms of Cu^{2+} in laccase *A*.[7] In order to get good resolution in the isoelectric focusing experiments the amount of protein used was low and hence the amount of the two components was too low to allow EPR studies and with this technique determine the state of Cu^{2+}. However, it has been shown earlier that only one of the Cu^{2+} ions is related to the strong blue colour and the unusual EPR spectrum [7] while the other Cu^{2+} ion gives no contribution to the blue colour. As both components found here are very blue they must contain the first type of Cu^{2+}. In addition it is most likely that both components also contain one atom Cu^{2+} of the second form. The reason for this is that EPR spectra of all preparations of laccase *A* shows that the second type of Cu^{2+} always is bound to the protein in the same manner which indicates a specific site for it.

Preliminary results from chromatographic fractionation of laccase *B* using the cation exchange SE-Sephadex C 50 and citrate buffers pH 5.0, I 0.005—0.05 indicated that there might be at least three protein components in this fraction although these components could not be completely separated. The isoelectric focusing investigation now done gave the result that laccase *B* was even more heterogeneous than laccase *A* and was resolved into ten components. Also these components must contain the first type of Cu^{2+} as at least the seven components present in highest amounts (those with pI 5.38—6.76) are visibly blue. As the ratio of the two forms of Cu^{2+} in laccase *B* also is 1:1 and EPR spectra show that the second type of Cu^{2+}, as in laccase *A*, is specifically bound, the concept that the enzyme molecule contains one Cu^{2+} of each form is further supported.

The reason why this enzyme has so many components cannot be decided at present time but a number of possibilities may be suggested. The heterogeneity might depend on a difference in amino acid composition but it is very improbable that the fungus would make twelve genetically different laccase molecules. However, as there are differences in activity among some of the components, they probably represent genetically different forms with at least a difference in amino acid composition at the active sites. The ultraviolet spectra of laccase *A* and *B* indicate a difference in the aromatic amino acid composition between these fractions.[6] Another explanation to the heterogeneity of laccase is that the components could have a difference in the carbo-

hydrate composition. In several glycoproteins the carbohydrates are in the form of rather long chains covalently bound to the protein part and the composition of these chains might be different as their manufacture supposedly is not genetically determined. This might explain why many glycoproteins are heterogeneous (for a review, see Refs. 11 and 12). The related protein ceruloplasmin has been found to be heterogeneous in electrophoresis.[11] As the isoelectric focusing method is more efficient, an investigation of ceruloplasmin with this technique might give even more components than earlier found.

The low pI of the two components of laccase *A* is related to the large excess of dicarboxylic acids over basic amino acids, amounting to 14 residues according to amino acid analysis of laccase *A*.[5]

The enzyme activities of the components, referred to the absorption at 280 nm, are rather different in laccase *B* whereas the two components of laccase *A* have similar activity. It was earlier found [5,6] that laccase *A* is more active than laccase *B*. The results from the present studies show that most components of laccase *B* are less active than the two components of laccase *A* but there are also components in laccase *B* (*e.g.* the component with the pI of 6.13) which have about the same activity as laccase *A*.

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