Determination of Copper Content in *o*-Diphenol Oxidase by Neutron Activation Analysis

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A polyphenol oxidase (*o*-diphenol oxidase) [*o*-diphenol: O₂ oxidoreductase E. C. 1.10. 3.1] from sweet potato named component II_b was highly purified. The copper content of this enzyme was measured by neutron activation analysis. Samples were analyzed with or without chemical separation after neutron irradiation. The copper content of the enzyme was determined to be 0.27%, and the minimum molecular weight of this enzyme was calculated to be 23,500.

Neutron activation analysis is suitable for determining small amounts of elements by measuring the radioactivity induced by neutron irradiation. This method has been recently applied to researches in various fields.^{1~3)}

o-Diphenol oxidases (Polyphenol oxidase, *o*-diphenol: O₂ oxidoreductase E. C. 1.10. 3.1) have been regarded as enzymes having copper atom at the active sites.^{4,5)} It is reasonable to assume, in the case of *o*-diphenol oxidases isolated from sweet potato (*Ipomea batatas*) roots, that they are also copper enzymes, because their activities are inhibited by copper chelating agents such as potassium cyanide, diethyldithiocarbamate and phenylthiourea.^{6,7)} Eiger and Dawson isolated *o*-diphenol oxidase from sweet potato and examined the copper

content in that enzyme.⁸⁾ Recently, Hyodo and Uritani isolated *o*-diphenol oxidases from sweet potato and purified one component among them,⁷⁾ the copper content of which was determined by neutron activation analysis using Japan Research Reactor No. 1 (JRR-1) in Japan Atomic Energy Research Institute. This paper deals with an analysis of the copper content of the enzyme by the activation analysis.

MATERIALS AND METHODS

Isolation and Purification of *o*-Diphenol Oxidases

o-Diphenol oxidases, also called polyphenol oxidase II,⁶⁾ were extracted from about 12 kg of healthy tissue of sweet potato roots with 0.05 M phosphate buffer (pH 7.3) and fractionated with ammonium sulfate. The fraction between 0.3 and 0.6 saturation was collected. This fraction was then subjected to DEAE-cellulose column chromatography, and the effluent showing *o*-diphenol oxidase activity was collected. Rechromatography of the active fraction through the DEAE-cellulose column was made in different conditions. From these chromatographic separations three definite peaks were obtained for *o*-diphenol oxidases. Thus, the enzymes, according to the individual peaks, were named as components II_a, II_b, and II_c correspondingly. II_b was collected and sepa-

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rated further by hydroxyapatite column to remove other proteins. By such manipulation, the specific activity of II_b was increased to about 520 times higher than that of the crude extract. An additional confirmation using an analytical centrifuge showed that the enzyme thus prepared could be regarded as a single protein component. The methods of the isolation and purification as well as several enzymological properties of these enzymes will be described in more detail elsewhere.

Preparation of the Samples for Activation Analysis

Component II_b, obtained as above mentioned, was dialyzed in deionized water. Exactly 0.6 ml and 0.8 ml of the enzyme preparation were placed on two separate sheets of filter paper (1.8×4.5 cm). The filter papers were then dried under a hot lamp. Two sheets of the filter paper containing the enzyme were put into individual polyethylene pouches, which were kept in a capsule (3×8 cm). Cupric sulfate was used for preparing a standard cupric aqueous solution, which contained 2 μg of cupric ion per ml. One ml of the standard solution placed on a sheet of filter paper was also kept in the above capsule. Another sheet of filter paper of the same size, which contained only deionized water, was used as a control.

Neutron Irradiation

The capsule containing the above mentioned samples was irradiated in JRR-1 reactor No. 16 experimental hole (Pneumatic tube, thermal neutron flux $3 \times 10^{14} n/cm^2 \cdot sec$) for 2 hr.

Chemical Separation of Copper Atoms after Neutron Irradiation

After irradiation the filter papers were taken out of the capsule, and to each of them was added 1 ml of carrier solution containing 1 mg of cupric ion. These filter papers were then dried and burned to ash in a crucible. Several drops of concentrated nitric acids were added to the ash, and the contents were dried by heating on a sand bath. After the samples were completely dry, several drops of concentrated hydrochloric acid were added. The samples were again heated to dryness and an addition of hydrochloric acid was repeated. Finally the residue in the crucible was dissolved in 20 ml of 2 N hydrochloric acid. A small amount of ascorbic acid was added to the solution, and the mixture was heated until the greenish-blue color of the solution disappeared. The solution was passed through an anion

exchange resin column (Diaion SA #100, analytical grade, 100~100 mesh Cl-type, column: 1×10 cm) which was equilibrated previously with 2 N hydrochloric acid solution containing ascorbic acid. The column containing the sample was washed with 20 ml of the same solution. The cuprous ion adsorbed in the form of a chloro-complex was changed to cupric ion and eluted by applying 30 ml of 2 N hydrochloric acid solution containing 0.8% hydrogen peroxide. About 30 ml, of the effluent was concentrated to a few ml, and the concentrate was made up exactly to 10 ml. This sample was divided into two equal parts. The first part was used for the radioactivity determination of the activated copper atoms and the second part for titration.

The pH of the second part was adjusted to 8.0 with ammonium water and ammonium chloride and titrated with 0.01 M ethylenediaminetetraacetic acid (EDTA). DOTITE MX was used as the indicator (yellow to red). A solution containing 500 μg of the cupric ion was also titrated in order to calculate the recovery of cupric ion after the above mentioned procedure of chemical separation.

The radioactivity were measured with gamma-ray scintillation counter (Aloka Model DC-5, well type NaI (Tl) crystal 1.3/4×2'') and 256-channel gamma-ray scintillation spectrometer (RCL Model 20609, well type NaI (Tl) crystal 1.3/4×2'').

Non-destructive Activation Analysis

After neutron irradiation, samples were directly subjected to 256-channel gamma-ray scintillation spectrometry without the chemical separation.

Determination of Protein Content of the Enzyme Solution

The protein content was calculated from nitrogen determination by Nessler's reagents after digesting with sulfuric acid.⁹⁾ The enzyme solution (0.3 ml) was mixed with 1 ml of 10 N H₂SO₄ containing CuSeO₃ and digested overnight. After digestion 2 ml of water, 5 ml of color reagent, and 3 ml of 5 N NaOH were added to the solution. Optical density at 497 mμ was measured after standing for 20 min. The protein content was estimated by the usual procedure of multiplying the nitrogen value by 6.25.

RESULTS AND DISCUSSION

The recovery of the cupric ion after the chemical separation was estimated by the

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EDTA-titration method. The results are shown in Table I, with C, St, S1, and S2 representing the control sample (1.0 ml of pure water and filter paper), the standard sample (2 μ g of cupric ion in 1.0 ml of pure water and filter paper), the enzyme sample 1 (0.6 ml of enzyme sol. absorbed on filter paper), and the enzyme sample 2 (0.8 ml of the same enzyme solution absorbed on filter paper), respectively. As described in Materials and Methods, each sample (5 ml) contained 500 μ g of cupric ion before chemically separated. S(Cu) indicates a solution which contained 500 μ g of cupric ion in pure water and was titrated directly without the chemical separation.

The radioactivity of radioactive isotopes which is mainly due to Cu^{64} is shown in Table II. The first (I) and second (II) columns show experimental values and values calculated as 100% recovery. The third column (III) indicates values in which counts of the control sample (C) in II were subtracted from each count in II of the three samples (St, S1,

S2). The copper content of each sample was calculated by dividing the count of the enzyme sample in column II by that of the sample St (6852 c.p.m.), and then multiplying this by the copper content of St which is 1.00 μ g. Thus, the copper content of S1 which contained 0.3 ml of the enzyme preparation was estimated to be 0.32 μ g. Consequently, 1.07 μ g of copper should be present in 1.0 ml of the enzyme preparation. By the similar calculation, S2 was found to contain 1.36 μ g of copper per 1.0 ml, and the average of these two samples is 1.22 μ g of copper per 1.0 ml of the enzyme preparation.

Another method, which uses γ -ray spectrometry, was also employed for determination of the copper content of the same samples mentioned above. The γ -ray scintillation spectra of C, St, S1, and S2 are shown in Fig. 1. A Cu^{64} peak located at the energy level of 0.51 MeV was found for all spectra. The area under the peak at 0.51 MeV was estimated by weighing out each individual peak which was cut out from the spectra. The calculation of the copper content in each sample was performed based on the 100% recovery as indicated in the previous section. The result of such calculation is presented in Table III. The first column shows the actual experimental values, and the second column shows the values calculated as 100% recovery. The copper contents of S1 and S2 were thus calculated by comparing with the copper content of St sample. The values are 1.13 μ g and 1.43 μ g per ml and the average is 1.28 μ g.

TABLE I. RECOVERY OF CUPRIC ION AFTER CHEMICAL SEPARATION

	EDTA Titration (ml)	Recovery (%)
C	5.45	75.7
St	5.00	69.4
S1	5.47	76.0
S2	4.28	59.4
S(Cu)	7.20	

C, St, S1, S2 and S(Cu) represent control sample, standard sample, enzyme sample 1, enzyme sample 2 and standard cupric solution containing 500 μ g of cupric ion, respectively.

TABLE II. DETERMINATION OF COPPER CONTENT OF THE ENZYME SAMPLE BY c. p. m. AND RECOVERY AFTER CHEMICAL SEPARATION

	I (c. p. m.) Exp. Value	II (c. p. m.) 100% Recovery	III (c. p. m.) -IIC	Copper content (μ g)	Copper Content of the enzyme preparation (μ g/ml)	Average (μ g/ml)
C	355	469				
St	5081	7321	6852	1.00		
S1	2035	2678	2209	0.32	1.07	1.22
S2	2487	4187	3718	0.55	1.36	

C, St, S1 and S2 represent control sample, standard sample, enzyme sample 1 and enzyme sample 2, respectively.

FIG. 1. γ -Ray Spectra of Samples after Chemical Separation.

C, St, S1 and S2 represent control sample, standard sample, enzyme sample 1 and enzyme sample 2, respectively.

Spectra of γ -scintillation spectrometry of the non-destructive samples, that is, the samples without the chemical separation are shown in Fig. 2. In these spectra, the peaks other than that of Cu^{64} were observed, due to the contamination of other elements. However, the Cu^{64} peak is still quite conspicuous in each spectrum of St, S1 and S2. Copper contents of S1 and S2 were determined from those spectra in the same way as described for the destroyed samples. As shown in Table IV, the copper contents of S1 and S2 are $1.30 \mu\text{g}$ and $1.41 \mu\text{g}$ per ml, and the average is $1.36 \mu\text{g}$. Table V shows the values of copper content per 1.0 ml of the enzyme preparation obtained by the three different means. The values ($1.36 \mu\text{g}$) obtained for the sample without chemical separation is approximately the same as those values ($1.22 \mu\text{g}$ and $1.28 \mu\text{g}$) determined by the other two different methods which employed complicated procedures of chemical separation. Therefore, it is concluded that fairly reliable data can be obtained directly with the non-destructive method. However, the average of the two values ($1.22 \mu\text{g}$ and $1.28 \mu\text{g}$) determined by the chemical separation method is $1.25 \mu\text{g}$ and this might be the more accurate representation of the copper content in 1.0 ml of the enzyme preparation.

Since one ml of the enzyme preparation was found to contain $468.7 \mu\text{g}$ protein (as calculated from its nitrogen content), the copper content of the enzyme protein was estimated to be 0.27% ($1.25/468.7 \times 100 = 0.27\%$). The

TABLE III. DETERMINATION OF THE ENZYME SAMPLES BY γ -RAY SPECTROMETRY AFTER CHEMICAL SEPARATION

	Weight (mg)	Weight at 100% recovery (mg)	-Weight of C (mg)	Copper content (μg)	Copper content of the enzyme preparation ($\mu\text{g}/\text{ml}$)	Average ($\mu\text{g}/\text{ml}$)
C	2.0	2.6				
St	49.0	70.6	68.0	1.00		
S1	19.5	25.7	23.1	0.34	1.13	1.28
S2	24.5	41.3	38.7	0.54	1.43	

C, St, S1 and S2 represent control sample, standard sample, enzyme sample 1 and enzyme sample 2, respectively.

FIG. 2. γ -Ray Spectra of Non-Destructive (without chemical separation) Samples.

St, S1 and S2 represent standard sample, enzyme sample 1 and enzyme sample 2, respectively.

TABLE IV. DETERMINATION OF COPPER CONTENT OF THE ENZYME SAMPLES BY γ -RAY SPECTROMETRY WITHOUT CHEMICAL SEPARATION

	Weight (mg)	Copper content (μ g)	Copper content of the enzyme preparation (μ g/ml)	Average (μ g/ml)
St	52.0	2.00		
S1	22.0	0.85	1.30	1.36
S2	27.0	1.04	1.41	

St, S1 and S2 represent standard sample, enzyme sample 1 and enzyme sample 2, respectively.

TABLE V. COMPARISON OF COPPER CONTENTS DETERMINED BY THREE DIFFERENT METHODS

	Copper content per one ml of enzyme solution (μ g)
By scintillation counter after chemical separation	1.22
By γ -ray spectrometry after chemical separation	1.28
By γ -ray spectrometry without chemical separation	1.36

copper content of this enzyme thus obtained is very similar to those of some other copper containing enzymes previously investigated,

such as tyrosinase,¹⁰⁾ ascorbate oxidase,¹¹⁾ laccase¹²⁾ and certain other blue protein.¹³⁾ The resemblance in copper content of the present enzyme to those other copper proteins is interesting and should be emphasized.

At present the minimum molecular weight of the enzyme, component II_b, is roughly estimated to be 23,500. The exact molecular weight is however not known, since at present stage there is no way to estimate the number of copper atom involved in one molecule of enzyme protein. As mentioned before, there are several *o*-diphenol oxidases, such as components II_a, II_c and III present in sweet potato roots besides component II_b. It is also of great interest to determine the copper contents of these enzymes for comparison purpose because they are isozymes of component II_b. This may be a way to understand the enzymological relationship between these isozymes.

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