Purification and Properties of Co^{2+} Requiring Heme Protein Having Lipoxygenase Activity from *Fusarium oxysporum*

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Co^{2+} -requiring heme protein having lipoxygenase activity, obtained from *Fusarium oxysporum* (FUSARIUM lipoxygenase) was extensively purified by ammonium sulfate precipitation, ion exchange chromatography on SP-Sephadex and gel filtration with Sephadex G-100. The final preparation achieved homogeneity by ultracentrifugation and SDS-polyacrylamide gel electrophoresis. The molecular weight was estimated at 12,000 to 13,000 on the basis of ultracentrifugation, SDS-polyacrylamide gel electrophoresis and gel filtration. FUSARIUM lipoxygenase contained 1 mole protoheme IX per mole enzyme, required Co^{2+} as a stabilizing factor and lost activity by treatment with heat or proteases. FUSARIUM lipoxygenase-catalyzed oxidation was proved to be different from the well-known soybean lipoxygenase-catalyzed oxidation and hemeprotein or cobalt-catalyzed oxidations in various respects including reaction velocity, substrate specificity and activation energy.

Soybean lipoxygenase first obtained as a crystalline enzyme by Theorell *et al.*^{1,2)} has been most thoroughly studied, and assumed to be an exceptional case of oxygenase because it was reputed to possess no metals or prosthetic groups. However, more detailed experiments recently revealed the presence of approximately 1 atom of non-heme iron per mole enzyme.^{3~5)}

In the previous paper,⁶⁾ we described the isolation of potent fungus for lipoxygenase-like enzyme production, identification of the fungus, optimal conditions of the enzyme production and some properties of the crude enzyme. We also reported that crude extract from mycelia revealed multiple component nature. As a result of further purification, we purified one of these enzymes to give homogeneity by various physicochemical methods.

The purified enzyme possesses 1 mole protoheme IX per mole enzyme protein as a prosthetic group and requires Co^{2+} as a stabilizing factor. The physicochemical properties of the enzyme are quite different from soybean

lipoxygenase.

In this paper, the purification procedures, enzymatic properties and physicochemical properties of the Co^{2+} requiring heme protein having lipoxygenase activity (FUSARIUM Lipoxygenase) are presented, and considerable emphasis will be given in DISCUSSION to the difference between autooxidation-promoting catalysts, such as hemeproteins and heavy metals, and FUSARIUM lipoxygenase.

MATERIALS AND METHODS

Culture conditions and preparation of the enzyme. *Fusarium oxysporum* MT-81-1 was cultured under restricted aerobic conditions in a 5-liter conical flask charged with 3 liters of the basal medium⁶⁾ (pH 8.0) containing 0.1% soybean flour and 90 ml of soybean oil with agitation of 150 rpm at 26.5°C for 4 days. The cell pellet obtained by filtration with No. 2 TOYO filter paper (Tokyo) was kept frozen at -20°C until use.

The crude extract was prepared as described in the RESULTS by homogenization, destruction, centrifugation to remove the cell debris and fractionation with ammonium sulfate.

Assay of lipoxygenase activity. Lipoxygenase activity was determined by the following three methods.

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Method 1 (Assay by oxygen electrode method). Enzyme activity was assayed at 25°C by measuring O_2 uptake with an YSI Clark Oxygen Electrode (Yellow Springs) placed in a thermostatically controlled vessel. The substrate, either 32 mM linoleic acid or 30 mM methyl linoleate in 50 mM Na_2HPO_4 -NaOH buffer (pH 12.0) containing 1% Tween 40, was freshly made everyday.⁶⁾

Method 2 (Assay by spectrophotometric method). Enzyme activity was also determined by measuring the increase of absorbance at 234 nm resulting from formation of hydroperoxide from linoleate.⁶⁾

Method 3 (Assay by bixin breaching method). The reaction mixture contained 1.5 ml of 0.65% bixin, carotenoid carboxylic acid, in absolute ethanol solution (a few drops of 0.1 N NaOH were added for storage). To this mixture 2 ml of enzyme solution was added at regular intervals, followed by addition of 0.1 ml of 10% lead subacetate solution and agitation. After cooling, the solution was centrifuged at $25,000 \times g$ for 10 min and the absorbance of the supernatant solution was measured at 455 nm against blank prepared at zero time by adding lead subacetate before the addition of enzyme solution.

Enzyme units. One unit of lipoxygenase activity corresponds to the initial reaction rate of consumption of 1 μ mole O_2 per min at 25°C when the O_2 concentration of the substrate at 25°C is 250 μ M. In the case of dilute enzyme solution there was an initial lag in oxygen consumption, followed by a linear decrease which started after 30 to 60 sec. All measure-

FIG. 1. Effect of Enzyme Concentration on Initial Velocity.

FUSARIUM lipoxygenase activity was measured at 25°C by assay method 1 with 30 mM methyl linoleate in 50 mM Na_2HPO_4 -NaOH buffer (pH 12) containing 1% Tween 40 as substrate.

ments were carried out in the linear portion of the oxygen consumption graph. Under these conditions the initial velocity was strictly proportional to the enzyme concentration. (Fig. 1).

Protein determination. Protein concentration was determined by the method of Lowry *et al.*⁷⁾ or by the micro-biuret method⁸⁾ with bovin serum albumine as standard.

Electrophoresis. Polyacrylamide gel disc electrophoresis was carried out on 15% and 30% acrylamide gels at pH 4.3 at 5°C and with a constant current of 1 mA per tube in accordance with the method of Ornstein and Davis.⁹⁾

When the gels were stained with the substrate and KI solution for observation of the lipoxygenase activity band by visualizing the hydroperoxide produced, they were modified to contain 1% starch¹⁰⁾ and 5% acrylamide to facilitate permeation of the substrate and KI.

Sodium dodecylsulfate (SDS) containing polyacrylamide gel electrophoresis was done on 17% and 12% gels at 5°C with a constant current of 8 mA per tube by the method of Weber and Osborn,¹¹⁾ and the molecular weight was also determined with control markers. The gels were stained with amido black and scanned at 600 nm with a Gilford Model 2400 recording spectrophotometer.

Isoelectric focusing. The isoelectric point was determined by isoelectric focusing electrophoresis¹²⁾ with a 110 ml capacity Ampholine Column (LKB Instruments, Inc.). The carrier ampholyte (Ampholine, LKB) was selected to give a pH gradient between 9 and 11, and used at an average final concentration of 1%. Other procedures were performed as described in the instruction manual.¹³⁾ Electrofocusing was performed for 72 hr with a final potential of 500 V at 5°C.

Molecular weight determination. Molecular weight was determined by the methods of Andrews¹⁴⁾ (gel filtration), Weber and Osborn¹⁴⁾ (SDS-polyacrylamide gel electrophoresis), and Archibald¹⁵⁾ (ultracentrifugation).

Ultracentrifugal analysis. A Hitachi UCA-1 analytical ultracentrifuge with synthetic boundary cells and an RA-60 rotor were used for the sedimentation analysis. The molecular weight was determined by the Archibald method¹⁵⁾ at 21,410 rpm (23.5°C).

Spectral analysis. The spectra of this enzyme were recorded with a Hitachi Model 124 doublebeam spectrophotometer at room temperature or a Cary model 14 recording spectrophotometer at 25°C.

Amino acid analysis. Purified protein preparations

were hydrolyzed in 6 N HCl in a fair vacuum at 110°C for 24, 48 and 72 hr, respectively. Amino acid analysis was carried out with a Nihon Denshi Model JLC-6AH automatic amino acid analyzer by the method of Spackman *et al.*¹⁰⁾

Contents of tryptophan and tyrosine were determined from the UV spectrum of the enzyme in 0.1 N NaOH.¹⁷⁾

Heme assay. The heme content of the homogeneous FUSARIUM lipoygenase was determined by the pyridine hemochromogen method^{15,19)} with a solution of hemin chloride freshly dissolved in dilute alkali used as a control.

Chemicals. Sephadex G-100, G-75 and SP-Sephadex C-25 were obtained from Pharmacia Fine Chemicals. Protein markers kit for determination of molecular weight was from Mann Res. Lab. Linoleic acid (above 99%) was from Applied Science Lab. Inc. and methyl linoleate (approx. 99%), from Sigma Chemical Co. Pancreatic lipase, *St. griseus* protease (Pronase P), *B. subtilis* proteinase (Nagase) and α -chymotrypsin were supplied by E. Merck, Kaken Kagaku Co., Ltd., Nagase Sangyo Co., Ltd., and Sigma Chemical Co., Ltd., respectively. Bixin was purchased from Tokyo Kasei Co., Ltd. The other chemicals were of reagent grade.

RESULTS

I. Multiple active components of crude extracts

The lyophilized crude extracts, 0.6 g, were dissolved in 30 ml of 50 mM phosphate buffer, pH 6.0. Then the crude enzyme solution was passed through the Sephadex G-100 column (5.0×90 cm). The elution rate was kept at 90 ml per hr, and fractions of 15 ml were collected.

The elution profile is shown in Fig. 2. The crude enzyme extracts separated into four major active fractions, I, II, III and IV. The whole protein recovery through this procedure was 75% and the fraction III revealed not only high recovery of activity but also the highest specific activity and reproducibility of production among these multiple active components.

II. Purification of FUSARIUM lipoygenase

We concluded from the results of experiment described above that the fraction III was the component to be purified among the multiple enzymes.

All operations were performed at zero to 5°C. The phosphate buffer used routinely in the purification procedure contained 0.25 mM CoCl₂ to stabilize the activity of the enzyme.

(Step 1) Preparation of cell extract. The frozen mycelia (wet 1500 g) were suspended in about 6 liters of 50 mM phosphate buffer (pH 6.0). This suspension was divided into adequate amounts and homogenized with NIHON SEIKI warring blender (Nihon Seiki Kogyo Co., Japan) for 1 min, and the cells were destroyed by the two-cycle treatment with a Manton-Gaulin (Manton-Gaulin Manufacturing Co., U.S.A.) at 8000 lbs. The cell debris was removed by centrifugation at 12000×g for 60 min (Fraction A, Table I).

(Step 2) Ammonium sulfate fractionation and freezing treatment. Solid ammonium

FIG. 2. Gelfiltration of the Enzyme Precipitated with Ammonium Sulfate on Sephadex G-100.

●---●, absorbance at 280 nm; ○—○, FUSARIUM lipoygenase activity.

sulfate was added to fraction A to 10% saturation, and the inactive material was removed by centrifugation at $12000\times g$ for 60 min. Solid ammonium sulfate was further added to the supernatant to complete saturation. After standing overnight with stirring in a cold room, the precipitate was frozen immediately and melted slowly at room temperature to denature contaminated proteins. After centrifugation at $12000\times g$ for 90 min, the precipitate was dissolved in 700 ml of 50 mM phosphate buffer, pH 6.0, and dialyzed for 24 hr against the same buffer 3 times. After the dialysis, the denatured materials were removed by centrifugation at $12000\times g$ for 30 min. The supernatant was concentrated by ultrafiltration through a DIAFILTER G-05T filter (Bioengineering Co., Tokyo). (Fraction B)

(Step 3) *1st column chromatography on SP-Sephadex.* SP-Sephadex C-25 was packed in a column (5×25 cm) equilibrated with 50 mM phosphate buffer, pH 6.0. The concentrated enzyme solution was adsorbed on the column and then washed with 1 liter of the same buffer. The adsorbed enzyme was subsequently eluted with 300 ml of 0.4 M sodium chloride in the same buffer. The eluate was pooled and dialyzed overnight against the same buffer, and concentrated by ultrafiltration as described in step 2. (Fraction C)

(Step 4) *Gelfiltration on Sephadex G-100.* A portion of the concentrated fraction C, 2 ml, was then passed through the Sephadex G-100 column (1.5×75 cm) previously equilibrated with the 50 mM phosphate buffer. The elution rate was kept at about 4 ml per hr, and fractions of 2.5 ml were collected. The fractions containing lipoxygenase activity were pooled and concentrated. (Fraction D)

(Step 5) *2nd column chromatography on SP-Sephadex.* The concentrated fraction D was adsorbed on a column (1.0×25 cm) of SP-Sephadex C-25 previously equilibrated with 50 mM phosphate buffer, pH 6.0. The lipoxygenase was eluted with a 200 ml linear gradient of increasing NaCl concentration from zero to 0.15 M. Fractions of 2.5 ml were collected. The enzyme which showed a single peak at step 4 produced one or two minor active peaks besides the main peak. The minor peak of activity eluted after the main peak was only occasionally observed as shown in Fig. 3, and the 2nd minor peak eluted after the first minor peak was very rare.

Various factors including specific activity and heme content suggested that the main active peak corresponded to a native protein and the other minor peaks, to denatured proteins.

Main active fractions were pooled (Fraction E) and stored at -80°C .

FIG. 3. Elution Profile of FUSARIUM Lipoxygenase on SP-Sephadex C-25 Column.

●---●, absorbance at 280 nm; ○---○, FUSARIUM lipoxygenase activity; ----, NaCl Concentration.

TABLE I. PURIFICATION PROCESS OF FUSARIUM LIPOXYGENASE

Fraction	Total protein mg	Total units ^{a)}	Recovery %	Specific activity Units/mg protein	Concentration
A. Crude extract	41,900	875	100	0.02	1
B. (NH ₄) ₂ SO ₄ fraction freezing treatment	6800	648	74	0.10	5
C. 1st SP-Sephadex	113	249	28	2.2	110
D. Sephadex G-100	34	225	26	6.6	330
E. 2nd SP-Sephadex	20	188	21	9.3	465

^{a)} The enzyme activity was determined by assay method 1 with methyl linoleate as substrate.

The results of purification procedures of FUSARIUM lipoxygenase (Fraction III) are summarized in Table I. FUSARIUM lipoxygenase was purified approximately 470-fold, and the purified enzyme was obtained at a yield of 20 mg per 1.5 kg wet mycelia. Fraction E was used in the following experiments.

III. Homogeneity of FUSARIUM lipoxygenase

As shown in Fig. 4, purified FUSARIUM

lipoxygenase formed only one band on polyacrylamide gel disc electrophoresis at pH 4.3 regardless of the concentration of acrylamide and on the gel electrophoresis in the presence of SDS.

When the gel was stained owing to the enzyme activity, only one band of activity was also observed. In this case, the lipoxygenase band was found to have a somewhat diffuse zone because of the low concentration of acrylamide. (Fig. 5)

The purified enzyme preparation showed a

FIG. 4. Polyacrylamide Gel Electrophoresis.

Polyacrylamide disc gel electrophoresis was performed in 15% acryl amide gel, pH 4.3, for 5 hr at a constant current of 1 mA per tube (A). SDS-polyacrylamide electrophoresis was performed in 12% acrylamide gel containing SDS at a constant current of 8 mA per tube (B). Both were carried out at 5°C and stained with a solution of amido black. The scanning profiles at 600 nm obtained with a Gillford Model 2400 recording spectrophotometer are also presented (C, D). The arrows show the tops of gels.

FIG. 5. Specific Staining of FUSARIUM Lipoxygenase.

Electrophoresis was performed in 5% acrylamide gel containing 1% starch, pH 4.3, for 5 hr at 5°C and at a constant current of 1.4 mA per tube. Specific staining was carried out with KI-starch, with linoleic acid as a substrate (A). The scanning profile at 580 nm obtained with a Gillford Model 2400 recording spectrophotometer is presented (B). The arrow shows the top of gel.

FIG. 6. Ultracentrifugational Pattern of Purified FUSARIUM Lipoxxygenase.

Protein solution (0.4%) in 0.1 M potassium phosphate buffer containing 2.5×10^{-4} M Co^{2+} , pH 6.0, was centrifuged at 55,430 rpm. Photographic prints of the schlieren sedimentation pattern were taken at 20-min intervals (left to right).

single and symmetrical moving peak on the ultracentrifuge analysis. (Fig. 6) The sedimentation coefficient, $s_{20,w}$, was calculated to be 1.33.

IV. Enzymatic properties of homogeneous enzyme

The stoichiometrical relationships among various assay methods. A strong correlation was obtained between the amount of consumed O_2 measured by assay method 1 and the amount of the produced conjugated double bond measured by assay method 2 at 234 nm,

FIG. 7. The Stoichiometrical Relationships among Various Assay Methods.

Enzyme activities were measured at 25°C by assay methods 1, 2, and 3 with methyl linoleate as substrate. ○—○, Method 1 (Assay by oxygen electrode method); △—△, Method 2 (Assay by spectrophotometric method); □—□, Method 3 (Assay by bixin breaching method).

assuming ϵ : $234 = 2.74 \times 10^4$ (Fig. 7). A degree of relative decrease of O.D._{455} measured by assay method 3, bixin breaching method, was also closely related to the above correlation. In conclusion, the assumption that the purified FUSARIUM lipoxxygenase produces one mole hydroperoxide from one mole substrate and one mole O_2 was ascertained.

Effect of pH. The effect of pH on the enzyme activity is shown in Fig. 8. An unusual sharp peak at pH 12 and an anomalous high optimum pH were obtained, whereas there was a shoulder at pH 10. This enzyme was most stable in the region of pH 8.0 to 9.0 as

FIG. 8. Effect of pH on Enzyme Activity.

The assay was performed by enzyme assay method 1 except that the reaction pH was varied as indicated.

shown in Fig. 9.

Effect of temperature. Thermostability of the enzyme was investigated at various temperatures as shown in Fig. 10. The enzyme was stable at 70°C for 15 min in the 50 mM

FIG. 9. Effect of pH on Enzyme Stability.

The enzyme (0.05 mg/ml) was allowed to stand at 40°C for 10 min in MacIlvaine buffer (0.1 M citric acid-0.2 M Na_2HPO_4) for pH 3~6, 0.1 M phosphate buffer for pH 6~8, 0.1 M Tris-HCl buffer for pH 8~10, and 0.1 M borate buffer for pH 10~12. Each buffers contained 2.5×10^{-4} M Co^{2+} as a stabilizing factor. The remaining activity was assayed by method 1. Average values were plotted in regions of overlapping buffers.

FIG. 10. Effect of Temperature on Enzyme Stability.

The enzyme (0.05 mg/ml) in 50 mM phosphate buffer (pH 8.0) containing 2.5×10^{-4} M Co^{2+} was kept at various temperatures for 15 min. After rapid cooling, the remaining activity was measured by assay method 1.

phosphate buffer (pH 8.0) containing 2.5×10^{-4} M Co^{2+} , whereas the activity was lost completely by heating treatment for 60 min at 100°C.

Effect of temperature on enzyme activity was studied and the Arrhenius plot of data is presented in Fig. 11. Activation energy of the enzyme reaction calculated from the slope of the line below 30°C was 11.4 kcal/mole. The optimum temperature for FUSARIUM lipoygenase reaction was 40°C.

FIG. 11. Arrhenius Plots for the Reaction of FUSARIUM Lipoygenase.

The assay was carried out by method 3 except that the reaction temperature was varied. Activation energy calculated below 30°C was 11.4 kcal per mole.

Stabilizing factor. In the previous paper, we described that Co^{2+} , Mg^{2+} and Cd^{2+} played a stabilizing role and Co^{2+} was always included in the buffer. The role of Co^{2+} as a stabilizing factor was examined in detail with regard to a homogeneous enzyme (Table II). When Co^{2+} was removed by dialysis against the same buffer free of Co^{2+} at 3°C, the enzyme maintained over 70% of the activity of the original enzyme before dialysis. On the other hand, when the dialyzed enzyme was heated at 30°C for 10 min in the absence of Co^{2+} it lost activity completely, whereas

TABLE II. EFFECT OF Co^{2+} ON THE STABILITY OF PURIFIED FUSARIUM LIPOXYGENASE

	Relative activity ^{a)}
Control	100
Dialysis against 50 mM phosphate buffer (pH 6.0) free of Co^{2+} and incubation at	
3°C for 24 hr	78
3°C for 48 hr	74
3°C for 72 hr	72
30°C for 10 min	100
{ + Co^{2+}	
{ - Co^{2+}	0
Addition of Co^{2+} to the dialyzed enzyme and incubation at 30°C for 30 min	
Co^{2+} Concentration (M)	
5×10^{-2}	9
5×10^{-3}	5
1×10^{-3}	0

^{a)} The enzyme activity was determined by assay method 1. In the case of addition of high concentration of Co^{2+} , the quantity of O_2 consumed by Co^{2+} alone as an autoxidation promoting catalyst was subtracted from the actual experimental values. Control signifies the preparation which was not dialyzed and not incubated.

100% activity was retained through the incubation in the presence of Co^{2+} (2.5×10^{-4} M). This disappearance of enzyme activity was not recovered to the previous level even if a large quantity of Co^{2+} was added.

In conclusion, FUSARIUM lipoxigenase requires Co^{2+} as an essential stabilizing factor.

Effect of various chemicals on enzyme activity. When the enzyme (0.05 mg/ml) was incubated with proteases (1 mg/ml), i.e., α -chymotrypsin, Nagase and Pronase, in 50 mM phosphate buffer (pH 7.5) containing 2.5×10^{-4} M Co^{2+} at 37°C for 3 hr, the activity measured by assay method 1 was lost by 56%, 82% and 44%, respectively.

The effect of various chelators on FUSARIUM lipoxigenase are summarized in Table III. Chelating agents, such as *o*-phenanthroline which is effective for Fe^{2+} , 8-hydroxyquinoline, thioglycollic acid and quinalizarin which are effective for Co^{2+} , caused 50 to 100% inhibition at 10^{-3} M. Inhibition by 70 to 90%

TABLE III. EFFECT OF VARIOUS SUBSTANCES ON ENZYME ACTIVITY

Enzyme (0.05 mg/ml) was incubated with chelators at 30°C for 30 min in 50 mM phosphate buffer (pH 7.3) and with Urea (6 M) at 37°C for 3 hr in 50 mM phosphate buffer (pH 6.0). Incubation systems contained 2.5×10^{-4} M Co^{2+} as a stabilizing factor and the activity was assayed by method 1. Enzyme (0.05 mg/ml) and such substances as cyanide, azide and fluoride in the substrate were preincubated separately and mixed at the moment when the assay was carried out by method 1.

Reagent	Concentration (M)	Inhibition (%)
α, α' -Dipyridyl	10^{-3}	12
EDTA	10^{-3}	0
Thioglycollic acid	10^{-3}	100
8-Hydroxyquinoline	10^{-3}	46
<i>o</i> -Phenanthroline	10^{-3}	77
Sodium diethyldithiocarbamate	10^{-3}	0
α -Benzoinoxime	10^{-3}	15
α -Nitroso- β -naphthol	10^{-3}	23
Phenylthiohydantoic acid	10^{-3}	29
Quinalizarin	10^{-3}	92
Iminodiacetic acid	10^{-3}	1
Quinaldic acid	10^{-3}	19
<i>p</i> CMB	10^{-3}	0
Urea	6	12
KCN	10^{-2}	40
NaN_3	10^{-2}	0
NaF	10^{-3}	0

was also observed with dithizone (1,5-diphenylthiocarbazone) and dithiol (toluene-3,4-dithiol), chelating agents which possess sulfhydryl group. On the other hand, Tiron (catechol-3,5-disulfonic acid) which is effective for Fe^{3+} did not cause appreciable inhibition, nor did ethylenediaminetetraacetate (EDTA), sodiumdiethyldithiocarbamate, *p*-chloromercuribenzoate and iminodiacetic acid. The other substances, such as urea, azide and fluoride, were ineffective, whereas cyanide was a highly effective inhibitor.

The enzyme (0.05 mg/ml) was not inactivated by the treatment of a pancreatic lipase (1 mg/ml) at 37°C for 3 hr in 50 mM Tris-HCl buffer (pH 8.5).

We conclude that the Co^{2+} plays a stabilizing role and Fe probably contained in a heme acts as a prosthetic group of FUSARIUM lipoxigenase.

Substrate specificity. The homogeneous enzyme also is fairly specific for linoleic acid and methyl linoleate as described in the previous paper.⁶⁾ The incubation mixture contained 32 mM fatty acid or its metnyl ester, and the initial rate of O_2 uptake was measured at 25°C as described for assay method 1. The specific activity of purified enzyme was 19.4, 9.5, 1.46 and 0.95 units/mg for linoleic acid, methyl linoleate, linolenic acid and methyl linolenate, respectively.

The monoenoic oleic acid and the saturated stearic acid were inactive. Then we concluded that a *cis*, *cis*-1,4-pentadiene structure in the substrate was essential for enzyme activity as soybean lipoyxygenase. But the specificity of the enzyme for unsaturated fatty acids was different from the results obtained with several plant lipoyxygenases²¹⁾ which indicate that linoleic acid and linolenic acid are equally effective substrates, and it also differed from autoxidation and hemeprotein or heavy metal-catalyzed oxidation which increase as a function of the unsaturation of the fatty acid.

The rate of oxidation of methyl linoleate by O_2 consumption at 25°C were 121, 1.1 and 2.3×10^{-2} moles O_2 /mole catalyst/min for FUSARIUM lipoyxygenase, hemin and $CoCl_2$, respectively. The rate of FUSARIUM lipoyxygenase-catalyzed oxidation is much greater

than that of hemin or $CoCl_2$ -catalyzed oxidations.

Michaelis constant. The michaelis constant for linoleic acid at pH 12 was determined to be 1.69×10^{-3} M according to the Lineweaver and Burk plot as shown in Fig. 12.

V. Physicochemical properties of homogeneous enzyme

Physicochemical properties are summarized in Table VI.

Molecular weight. The molecular weight estimated from the results of Sephadex G-75 gel filtration (Fig. 13) and SDS-polyacrylamide gel electrophoresis was 12,000 to 13,700. The molecular weight calculated by the Archibald method, based on a partial specific volume of 0.74, was 13,100 at the meniscus and 12,200 at the bottom.

FIG. 12. Double Reciprocal Plot of Linoleic Acid (substrate) Concentration vs. Initial Reaction Velocity.

The reaction was carried out at 25°C in the same manner as assay method 1 except that the substrate concentration was varied. The K_m was calculated to be 1.69×10^{-3} M.

FIG. 13. Plots of Elution Volume, (V_e), against Log (molecular weight) for Proteins on Sephadex G-75 Columns (1.5 × 80 cm) Equilibrated with 0.05 M Tris-HCl Buffer (pH 7.5) Containing 0.1 M KCl.

The diffusion coefficient ($D_{20,w}$) of the FUSARIUM lipoyxygenase was calculated from the data of the Archibald method to be 9.82×10^{-7} cm²sec⁻¹. Substitution of values of the sedimentation coefficient and the diffusion coefficient into the Svedberg equation²²⁾ gives a molecular weight of 12,800 for the enzyme. With the use of the experimentally proved data of $S_{20,w}$, $D_{20,w}$ and 13,000 for the molecular weight, the molar frictional ratio, f/f_0 ,²³⁾ of the enzyme was calculated to be 1.38. Assuming

that the enzyme molecule is not hydrated and the molecular shape is a prolate ellipsoid, a value of 8.1 can be calculated for the axial ratio, p .²³⁾ This value signifies a fairly wide discrepancy of the enzyme molecule from globular shape.

Absorption spectra. The absorption spectra of both the oxidized (native) and the dithionite-reduced forms of the purified FUSARIUM lipoygenase in the 50 mM phosphate buffer

FIG. 14. Absorption Spectra of FUSARIUM Lipoygenase.

Protein concentration was 0.1 mg/ml in 50 mM phosphate buffer, pH 6.0, containing 2.5×10^{-4} M Co^{2+} . A more detailed absorption spectrum of 280 nm region is presented in the inserted graph. -----, oxidized form; —, reduced form.

(pH 6.0) containing 2.5×10^{-4} M Co^{2+} are shown in Fig. 14. These represent typical absorption spectra of a heme protein having an absorption maximum at 413 nm and two broad peaks at 530 nm and 560 nm in the ferric state and maxima at 423 nm, at 525 nm with shoulders around 530 nm, 552 nm and 561 nm in the ferrous state. The broad α peaks in the ferric state split into the two sharp α peaks in the ferrous state.

The UV absorption pattern is not peculiar to ordinary proteins which have a peak around 280 nm, and the wavelike absorption pattern is observed around 260 nm as seen in superoxide dismutase.²⁴⁾

Specific absorption at 280 nm or 413 nm is presented in Table VI.

Identification of heme and measurement of heme content in the enzyme. Identification of the heme in the homogeneous enzyme was carried out after reduction with dithionite by the pyridine hemochromogen method^{18,19)} to the final concentrations of 8% (v/v) pyridine and 0.17 N sodium hydroxide. The spectra of the resultant pyridine hemochrome were compared with those of authentic heme as shown in Table IV. The absorption maxima and the ratios of extinctions, β/α and γ/α , of the enzyme were almost identical to those of hemin chloride and hematin, and we therefore concluded that the heme in FUSARIUM lipoygenase was protoheme IX.

The content of heme was determined with a value of 32 mM cm^{-1} for the absorption coefficient of the α band of the pyridine hemochrome at 557 nm.¹⁸⁾ The enzyme protein was found to contain 1 mole of the heme per mole of the enzyme, assuming 13,000 as the mole-

TABLE IV. ABSORPTION MAXIMA AND RATIOS OF EXTINCTIONS OF THE PYRIDINE FERROHEMOCHROME

The measurement was carried out with a Cary model 14 recording spectrophotometer at 25°C.

Source	α -band	β -band	γ -band	β/α	γ/α
	nm	nm	nm		
FUSARIUM lipoygenase	556.1	526	418	0.495	4.4
Hemin chloride	556	524	419	0.493	5.16
Hematin	556	526	418	0.61	4.4

TABLE V. AMINO ACID COMPOSITION OF HOMOGENEOUS FUSARIUM LIPOXYGENASE

Amino acid residue	Number	
	Calculated value ^{a)}	Nearest integer
Lysine	11.72	12
Histidine	2.51	3
Arginine	1.15	1
Aspartic acid	9.64	10
Threonine	2.58	3
Serine	4.82	5
Glutamic acid	6.93	7
Proline	5.66	6
Glycine	6.86	7
Alanine	3.68	4
Half-cystine	—	1
Valine	5.70	6
Methionine	0.41	1
Isoleucine	2.71	3
Leucine	3.83	4
Tyrosine	1.94	2
Phenylalanine	2.69	3
Tryptophan ^{b)}	1.16	1
Total		79

^{a)} Mean values for 24, 48 and 72 hr hydrolysates assuming MW 13,000.

^{b)} Determined spectrophotometrically by the method of Goodwin and Morton.¹⁷⁾

cular weight based on several methods described above.

Amino acid composition. The amino acid compositions of FUSARIUM lipoxygenase obtained as the average of 24 hr, 48 hr and 72 hr hydrolysates are summarized in Table V. The result shows that the enzyme has 79 amino acid residues per mole and that it is rich in lysine and aspartic acid. The molecular weight was determined to be 11,000 on the basis of amino acid and heme content.

Isoelectric point. The isoelectric point of FUSARIUM lipoxygenase was calculated to be 9.46 by isoelectric-focusing electrophoresis.

DISCUSSION

Co²⁺-requiring heme protein having lipoxygenase activity isolated from *Fusarium oxysporum* MT81-1 (FUSARIUM Lipoxygenase) was purified to homogeneity. It is well known that naturally occurring compounds, especially heavy metals and heme compounds, catalyze the peroxidation of unsaturated lipids. In fact,

TABLE VI. PHYSICOCHEMICAL PROPERTIES OF FUSARIUM LIPOXYGENASE

Sedimentation coefficient (sec $\times 10^{13}$)	$S_{20,w}$	1.33
Diffusion coefficient (cm ² sec ⁻¹ $\times 10^7$)	$D_{20,w}$	9.82
Molar frictional ratio	f/f_0	1.38
Axial ratio (assuming prolate ellipsoidal model)	p	8.1
Molecular weight		
Svedberg		12,770
Archibald	(meniscus)	13,130
	(bottom)	12,230
Andrews		12,000
SDS-polyacrylamide		13,700
Amino acid analysis		10,160
	(approx.)	10,840
Isoelectric point		9.46
Protoheme to protein, molar ratio		1
Specific absorbance	$E_{1\text{cm}}^{0.1\%}$	
280 nm		1.14
413 nm		5.5
Absorptivity	ϵ MM	
280 nm		14.3
413 nm		68.8
Turnover number	(methyl linoleate)	121
(moles of O ₂ consumed per min per mole of enzyme at 25°C)	(linoleic acid)	247
K_m (M)	(linoleic acid)	1.69×10^{-8}
Activation energy (Kcal/mole)		11.4

numerous workers have misinterpreted oxidation of unsaturated fatty acid by autoxidation promoting catalysts as the action of a lipoxxygenase. In the previous paper,⁶⁾ we described the high specificity for linoleate and the presence of Co^{2+} as a stabilizing factor to be indispensable culture conditions for the optimal formation of FUSARIUM lipoxxygenase.

These properties led us to distinguish between FUSARIUM lipoxxygenase and non-enzymatic oxidation catalysts.

In addition to these differences, the further distinction are clearly drawn from the following reasons:

1) Eriksson *et al.*²⁶⁾ reported that denaturation of heme proteins, such as catalase and peroxidase, resulted in increase of nonenzymatic activity of lipid oxidation concomitant with loss of enzymatic activity.

They proposed that the increased nonenzymatic activity was due to exposure of the catalytically active heme group. This phenomenon is in contrast to our results; namely, FUSARIUM lipoxxygenase loses enzyme activity with a treatment with heat or proteases. If FUSARIUM lipoxxygenase were a mere heme protein which catalyses the oxidation non-enzymatically, our results would not have been obtained.

2) Tappel²⁶⁾ reported that the activation energies for linoleates of both hemoglobin and cyt. C were 3.3 kcal/mole. When the activation energy of FUSARIUM lipoxxygenase is com-

pared with that of autoxidation promoting catalysts as shown in Table VII, the former is greater than the latter. Activation energies for linoleate were also reported to be 4.3, 6.5 and 8.6 kcal/mole for soybean lipoxxygenase,²⁷⁾ wheat lipoxxygenase²⁸⁾ and *Pseudomonas* lipoxxygenase-like enzyme,²⁹⁾ respectively. The higher activation energy of FUSARIUM lipoxxygenase than any other reported values of lipoxxygenases means high dependency of the enzyme reaction on temperature and seems to be reasonable for the enzyme-catalyzed reactions, most of which have activation energy of about 10 kcal/mole.

3) Tappel³⁰⁾ reported that the oxidation rate in hemoglobin-catalyzed linoleate oxidation increased over 2 fold between pH 7.8 and pH 9.5, whereas the pH-dependency of FUSARIUM lipoxxygenase-catalyzed reaction was much higher than that of hemoglobin as shown in Fig. 8. Opt. pH in FUSARIUM lipoxxygenase-catalyzed reaction is 12, and this value is different from that of heme proteins, 4.0 to 5.0,³¹⁾ hemin, under 6.96,³²⁾ or *Pseudomonas* lipoxxygenase-like enzyme, 10.8 to 11.0.²⁹⁾

4) The rate of oxidation catalyzed by FUSARIUM lipoxxygenase is much greater than that of hemin- or cobalt-catalyzed oxidation; *i.e.*, the ratio of oxidation of methyl linoleate per mole catalyst at 25°C is 5.3×10^4 : 4.8×10^2 : 1 for FUSARIUM lipoxxygenase, hemin and CoCl_2 in that order. Tappel²¹⁾ also reported that the ratio of oxidation of linoleate per mole catalyst at 0°C is 10^6 : 10^3 : 1 for plant lipoxxygenase, hematin compounds and copper in that order and this value comes near to our results. Purified FUSARIUM lipoxxygenase also differs from soybean lipoxxygenase in various respects, such as possession of protoheme IX as a prosthetic group and requirement of Co^{2+} as a stabilizing factor. The physicochemical and enzymatic properties of soybean lipoxxygenase,³³⁾ such as the molecular weight of 102,000, pI of pH 5.4, activation energy of 4.3 kcal/mole, opt. pH of 6.5 to 7.0, absence of inhibition by cyanide and oxidation of linoleic acid and linolenic acid at equal rates, are quite different from the pro-

TABLE VII. COMPARISON OF ACTIVATION ENERGIES

The activation energies were calculated in the same manner as in the case of FUSARIUM lipoxxygenase. CoCl_2 (0.1 M) in 0.01 M Tris-HCl buffer (pH 8.0), Hemin (0.1 mg/ml) in 0.01 N NaOH and crystalline horse heart Cyt. C (1 mg/ml) in 0.05 M phosphate buffer (pH 7.2) were used.

Catalyst	Reactant	Activation energy (Kcal/mol)
Hemin	Linoleic acid	5.0
Hemin	Methyl linoleate	2.1
Horse heart Cyt. C	Linoleic acid	4.4
CoCl_2	Methyl linoleate	4.1
F. lipoxxygenase	Methyl linoleate	11.4

properties of FUSARIUM lipxygenase except that the absence of inhibition by some chemicals, such as azide, fluoride, diethyldithiocarbamate and EDTA, is a common property, and the value of K_m (3×10^{-3} M) is approximately equal to that of FUSARIUM lipxygenase.

The unusual sharp peak at pH 12 and the anomalous high opt. pH were obtained in the study of pH-dependency of FUSARIUM lipxygenase activity. It must be taken into consideration that the solubility of the substrate increases with increasing alkalinity. So it was considered that these phenomena may be explained by the solubility of the substrate and the true opt. pH for the enzyme.

The one or two minor peaks observed on the stage of 2nd SP-Sephadex column chromatography seemed to be denatured proteins of the enzymes brought about by long storage. This is endorsed by several findings, namely, the increase of the number of minor peaks in proportion to the length of period of storage at -20°C , their decreased specific activity and decreased α -helix content measured by dichroic studies. To avoid the denaturation, storage of the enzyme preparation at -80°C in place of -20°C is necessary. The full verification of the denaturation process will be presented in the paper which will be published in the near future.

It is interesting that the α -peak was split into two peaks by the addition of dithionite. The phenomena of split of α peak that occurred in the ferrous state were also observed in b-type cytochromes at -190°C and at room temperature by Cheah³⁴⁾ and Yamanaka *et al.*,³⁵⁾ respectively. But the space of split peaks in FUSARIUM lipxygenase (9 nm) is wider than in these two earlier cases (7.5 and 7.0 nm).

From these results described in this paper, we concluded that FUSARIUM lipxygenase was a novel example of oxygenase containing a protoheme IX. It is noteworthy that this type of oxygenase which is known today is only tryptophan pyrrolase.^{36, 37)}

Other enzymatic properties are currently

under investigation

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