Physical and Chemical Properties of Crystalline Acetylcsterase from *Sclerotinia* Fungus

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The molecular weight of crystalline acetylcsterase (AcE) from *Sclerotinia libertiana* was determined to be 78,500 by both Archibald and gel filtration methods. The sedimentation coefficient, $s_{20,w}^0$, was determined to be 5.05 S. The amino acids and carbohydrate composition of AcE was determined as follows: Lys₁₂ His₁₄ Arg₁₈ Asp₇₆ Thr₅₂ Ser₆₂ Glu₆₀ Pro₄₈ Gly₉₀ Ala₅₇ Cysteine₄ Val₃₆ Met₈ Ile₃₇ Leu₄₂ Tyr₃₈ Phe₃₅ Trp₁₉ (carbohydrate as glucose)₁₆. The amino and carboxyl terminals of AcE were found to be threonine and serine, respectively. Isoelectric focusing electrophoresis with carrier ampholite revealed that AcE has an isoelectric point at around 3.95. Optical rotatory dispersion measurements showed that $[a]_D$, a_0 , b_0 and λ_c were -22 , -110 , -75 and 234.5 nm, respectively, suggesting that the enzyme contains 11% of the α -helix structure.

In a previous paper, we reported the purification method and some enzymatic properties of acetylcsterase (AcE, EC 3.1.1.6) of *Sclerotinia libertiana* FCL.¹⁾ The structural features of the esteratic site of AcE were suggested by the inhibition observed by certain kinds of polybasic organic acids as well as by the mechanism of action on the substrate having one or two ester-bonds in a molecule.

In the present paper, the physical and chemical properties of the crystalline AcE of *Sclerotinia libertiana* FCL were investigated.

MATERIALS AND METHODS

Enzyme. AcE of *Sclerotinia* fungus was purified by the method described previously,¹⁾ and the homogeneity of purified AcE was confirmed by electrophoresis, ultracentrifugation and chromatography on CM cellulose column. The specific activity was 2380 units per mg protein. The enzyme concentration was determined spectrophotometrically, assuming that the $E_{1\text{cm}}^{1\%}$ at 280 nm was 23.24. AcE activity was assayed according to the method described previously.¹⁾

Ultracentrifuge runs. Sedimentation velocities were measured with a Hitachi model UCA-1 ultracentrifuge operating at 54,400 rpm. The sedimentation coefficient was calculated as described by Schachman²⁾ and corrected to 20°C in water according to the method described by Svedberg.³⁾

Gel filtration. The molecular weight of AcE was estimated by Sephadex G-200 column chromatography according to Andrew's method.⁴⁾ The elution volume (V_e) was calibrated for estimating the molecular weight of proteins using proteins of known molecular weights as standards.

Isoelectric focusing electrophoresis. The carrier ampholytes (LKB-Produkter AB) used in this investigation were selected to give a pH gradient between pH 3 and 10. Focusing of the protein was carried out at 4°C in a special vertical electrolysis column of 110 ml capacity, equipped with a cooling jacket (LKB-Produkter AB). Measurements of pH were made with a Hitachi model M-5 pH meter.

Determination of amino acid composition. Ten milligrams of freeze-dried enzyme were hydrolyzed with 6 N hydrochloric acid in evacuated, sealed tubes at 110°C for 18, 24, 48 and 72 hr. In all cases small amounts of humins were formed during the hydrolysis. The hydrolysates were evaporated to dryness *in vacuo* to remove HCl and subjected to amino acid analysis by a Hitachi Amino Acid Autoanalyzer KLA-2. Values for serine and threonine were obtained by extrapolation to zero time, and maximum values were taken for isoleucine and arginine. The average of four periods of hydrolysis were used for the remainder of the amino acids. For the determination of tryptophan and tyrosine contents, the enzyme was dissolved in 1/10 N sodium hydroxide to the concentration of 5×10^{-5} M. The optical density of the enzyme solution was determined at 280, 294.4, 330 and 360 nm. The concentrations of tryptophan plus tyrosine were determined by

the routine method described by Goodwin and Morton.⁵⁾ The sulfhydryl groups were titrated spectrophotometrically with PCMB according to a modification of Boyer's method.⁶⁾ Half-cystine and methionine were determined as cysteic acid and methionine sulfone, respectively, after oxidation of the enzyme with performic acid as described by Hirs.⁷⁾

Detection of carbohydrate. The amounts of carbohydrate in AcE protein were determined by the phenol-sulfuric acid⁸⁾ and anthrone methods.⁹⁾ For the calculation, a standard curve was prepared with D-glucose.

N-terminal amino acid analysis

a. Dinitrophenyl method. The procedure of Fraenkel-Conrat, Harris and Levy¹⁰⁾ was used for N-terminal amino acid.

b. Detection by aminopeptidase. AcE protein was incubated with aminopeptidase of *Bacillus subtilis* under the conditions described by Minamiura *et al.*¹¹⁾ At certain time intervals, 10- μ l aliquots of the reaction mixture were taken and spotted on a filter paper. In order to stop the enzyme reaction, the spotted area was immediately acidified with a trace amount of 2.0 M acetic acid and dried in an air current. Paper chromatography was done using Toyo Roshi No. 50 paper with butanol: acetic acid: water (4: 1: 2) and butanol: pyridine: water (1: 1: 1) as developing solvents. The color density developed with ninhydrin was read on a densitometer, Toyo Densitorol DMU-2, and from the value, the quantity of amino acid was calculated by correcting with a factor obtained from paper chromatography with known quantities of amino acids.

C-terminal amino acid analysis

a. Detection by carboxypeptidase A. AcE protein was incubated with carboxypeptidase A of bovine pancreas purchased from Seikagaku Kogyo Co., according to the method described by Fraenkel-Conrat, Harris and Levy.¹⁰⁾ At certain time intervals, 10- μ l aliquots of the reaction mixture were taken and immediately acidified with a trace amount of 2.0 M acetic acid in order to stop the enzyme reaction. After being dried under N₂ gas flow, the residue was dissolved in a very small amount of buffer and subjected to analysis of C-terminal amino acid in a Hitachi Amino Acid Autoanalyzer KLA-2.

b. Detection by carboxypeptidase Y. AcE protein (3.5 mg) was dissolved in 20 μ l of 0.1 M pyridine-acetate buffer, pH 5.5, and digested at 25°C by carboxypeptidase Y.¹²⁾ The carboxypeptidase Y used was a sample supplied through the courtesy of Dr. Heinrickson, Department of Biochemistry, the University of Chicago. The weight ratio of AcE protein to carboxypeptidase Y was 250. Hydrolysis was stopped by adding 2.0 M

acetic acid at appropriate time intervals. The pyridine acetate buffer was removed in N₂ gas flow, then the samples were dissolved in pH 2.2 buffer and applied to an amino acid analyzer, Durrum D-500, after removal of insoluble material by centrifugation (range 1 to 10 nmoles per amino acid).

Measurement of optical rotatory dispersion (ORD). ORD measurement was done with a Jasco spectropolarimeter model ORD/UV-5 at the Osaka Municipal Technical Research Institute. The resulting data were interpreted by means of equation (1) of Moffitt-Yang.¹³⁾

$$[m'] = \frac{a_0 \lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0 \lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \quad (1)$$

The parameters, a_0 and b_0 , were evaluated from the intercept and slope, respectively, of a plot of $[m']_\lambda \frac{\lambda^2 - \lambda_0^2}{\lambda_0^2}$

versus $\frac{\lambda_0^2}{\lambda^2 - \lambda_0^2}$ where 212 nm was adopted as λ_0 . The dispersion constant, λ_c , in equation (2)

$$\lambda^2 [a]_\lambda = \lambda_c^2 [a]_\lambda + A \quad (2)$$

was calculated from the slope of the Yang plot, $\lambda^2 [a]_\lambda$ versus $[a]_\lambda$. ORD data in the ultraviolet region were measured with 0.4~1.0% of protein concentrations using cells of 0.1 mm path length and expressed in terms of $[m']$, the reduced mean residue rotation, defined as

$$[m'] = \frac{3}{(n^2 + 2)} \frac{m_0}{100} [a]_\lambda \quad (3)$$

where $[a]_\lambda$ is the specific rotation at wavelength λ , M_0 is the average residue weight (assumed to be 112), and n is the refractive index of the solvent at wavelength λ . The refractive indices as a function of wavelength were obtained from the table of Fasman. ORD measurements in the visible region were made from 300 to 600 nm using a cell of 0.5 cm path length. The protein concentration used was 0.78%.

RESULTS AND DISCUSSION

1. Crystallization of AcE

Acetone was cautiously added to the purified enzyme solution (1.5 ml) containing 1/200 M calcium acetate until a faint turbidity appeared at pH 6.0, and the amorphous precipitate was removed by centrifugation. The enzyme crystallized from the supernatant solution when it was allowed to stand overnight at 4°C. Figure 1 shows a microphotograph of crystalline AcE of *Sclerotinia* fungus which shaped triangle. The specific activity of the crystalline enzyme was 2380, and activity recovery was

FIG. 1. Microphotograph of Crystalline AcE.
($\times 600$)

about 40% of the original activity.

2. Physical properties of AcE

a. Sedimentation coefficient

Experiments were done at 34,400 rpm in a standard 12 mm, 4° sector cell. The sedimentation coefficients, $s_{20,w}$, at various concentrations of AcE protein in phosphate buffer at pH 7.0 of ionic strength 0.2 with sodium chloride, were calculated from the sedimentation patterns, typical one of which was presented in a previous paper.¹⁾ The sedimentation coefficient were obtained by extrapolating the plot of S versus concentration of the enzyme to zero, and the value of 5.5 S was obtained. A small variation of sedimentation coefficient was observed in accordance with the solvent employed, that is, $s_{20,w}^0$ was 5.05 S in acetate buffer at pH 5.5 of ionic strength 0.1, as shown in Fig. 2.

In the case of pig liver carboxylesterase,¹⁴⁾ the dissociation of native form to an active half molecular form occurs between pH 5.0 and 4.0, and the sedimentation coefficients change from 8.12 S to 4.1 S. In our case, sedimentation coefficient of AcE decreased slightly from 7.2 S in phosphate buffer at pH 7.0 of ionic strength 0.1 to 5.05 S in acetate

FIG. 2. Plots of Sedimentation Coefficient as a Function of AcE Concentration.

The individual $s_{20,w}$ values were obtained from runs in 0.0054 M acetate buffer, pH 5.5, with an ionic strength of 0.1 at 20°C . The extrapolated value of $s_{20,w}^0$ is 5.05 S.

buffer, at pH 5.5. The change of sedimentation coefficients may be related to the dissociation of native molecule to a half molecule, and this will be investigated under various conditions in further experiments. However, further experiments are required to manifest if this decrease in the sedimentation coefficients would be due to the dissociation of the native enzyme molecule.

b. Molecular weight

i. *Archibald method.* Approach to sedimentation equilibrium for the determination of molecular weight by Archibald method was conducted in a 12 mm, 4° sector cell at 13,100 rpm and at 23.5°C with 0.2 and 0.98% enzyme dissolved in the same buffer used for the sedimentation velocity runs. The concentration of original sample was determined in arbitrary units in a 12 mm, 4° sector synthetic boundary cell and average molecular weight at the meniscus was calculated according to the method described by Schachman.²⁾ The molecular weight of AcE in phosphate buffer at pH 7.0 of ionic strength 0.2 was calculated as approximately 78,500.

ii. *Gel-filtration.* The molecular weight of AcE was estimated by gel-filtration method with a Sephadex G-200 column. The molecular weight of AcE was estimated to be 78,500, as presented in Fig. 3. From the results of molecular weight estimation on

FIG. 3. Estimation of Molecular Weight of Crystalline AcE with Sephadex Gel Filtration.

The column (2.8×58 cm) of Sephadex G-200 was equilibrated with 0.75 M NaCl containing 0.025 M MgCl_2 . A, cytochrome *c* (horse); B, serum albumin (bovine); C, acetylcholinesterase; D, lactate dehydrogenase; E, γ -globulin.

equilibrium sedimentation and gel-filtration, AcE protein apparently has a molecular weight of 78,500. Recently, the molecular weight of pig liver esterase¹⁴⁾ has been shown to be 85,000~90,000 and that of rat liver esterase,¹⁵⁾ which has a trimeric structure, 177,000. The molecular weight of AcE is close to that of pig liver esterase.

c. Intrinsic viscosity

After determining the outflow times required by the buffer and protein solutions, the relative viscosity was calculated by the method described by Kuroiwa *et al.*¹⁶⁾ As shown in Fig. 4, the reduced viscosity was expressed as a function of protein concentration, and the intrinsic viscosity ($[\eta]$) was obtained as 0.0277 dl/g from the figure.

d. Partial specific volume

Duplicate determination of partial specific volume of AcE yielded a value of 0.724. The partial specific volume 0.726, evaluated from the amino acid composition, was slightly higher than the value determined by experiment.

The frictional ratio (f/f_0) was calculated to be 1.28 from sedimentation and molecular weight.

FIG. 4. Plot of Reduced Viscosity (η_{sp}/c) of Crystalline AcE as a Function of Protein Concentration.

Viscosity measurements were made in 0.005 M phosphate buffer at pH 7.0, with an ionic strength of 0.2 at 20°C. The intrinsic viscosity obtained was 0.0277 dl/g.

e. Isoelectric point

With 1.5 ml of enzyme solution, containing 25 mg of protein, an experiment of isoelectric focusing was performed. After focusing for 44 hr with a constant potential of 800 volts and an initial current of 4.8 mA, the contents of the column were cut into 3 ml fractions. The amounts of protein in the fractions were estimated by measuring absorbancy at 280 nm. On the basis of isoelectric focusing, AcE appeared to be a homogeneous substance having a pI at 3.95 (Fig. 5).

f. Optical rotatory dispersion measurement

The parameters of a_0 and b_0 were calculated from the Moffitt-Yang equation using 212 nm as λ_0 . The a_0 value was -110 and b_0 value -75 . Figure 6 shows ORD spectrum for the 195 to 270 nm region. AcE protein showed the negative Cotton effect at 232 nm with the value of 2200. Shoulder also appeared at 210 to 215 nm, suggesting the presence of the α -helix. AcE protein contains 11% of the α -helix structure judging from the b_0 value.

3. Amino acid composition

Amino acid composition of AcE of *Sclerotinia* fungus is presented in Table I. When

TABLE I. AMINO ACID COMPOSITION OF *Sclerotinia* FUNGAL ACETYLESTERASE

The values without any signs were the average of analyses of four hydrolysates obtained from 18,24,48, and 72 hr hydrolysates.

Amino acid	Amino acid residues	No. of amino acid residues ^{a)}	
	(%)	Calculated	Integer
Lysine	2.08	12.3	12
Histidine	2.56	14.2	14
Arginine	3.84	18.4	18
Aspartic acid	11.6	76.3	76
Threonine ^{b)}	7.06	51.7	52
Serine ^{b)}	6.90	61.7	62
Glutamic acid	10.25	60.2	60
Proline	6.17	48.2	48
Glycine	6.76	90.0	90
Alanine	5.32	57.0	57
Cysteine	0.56	4.0	4
Valine ^{c)}	4.69	36.1	36
Methionine	1.36	7.85	8
Isoleucine ^{c)}	5.53	37.0	37
Leucine	6.35	42.3	42
Tyrosine	8.21	38.4	38
Phenylalanine	6.72	34.7	35
Tryptophan ^{d)}	4.65	19.4	19
Total	100.61		708
NH ₃ ^{e)}	(1.03)	(49)	(49)

a) Based on a molecular weight of 78,500 and 3.34% of hexose content.

b) Values extrapolated to zero hydrolysis time.

c) Maximum values were adopted.

d) Value determined spectrometrically.

e) Determined only in the 18 hr hydrolysate.

FIG. 5. Isoelectric Focusing Pattern of Purified AcE.

Purified AcE, 25 mg, was used. Electrophoresis was carried out with an ampholyte of pH range from 3 to 6 with a current of 4.8 mA for 48 hr at 4°C. The column volume was 110 ml.

Δ—Δ, protein, absorbance at 280 nm; ○—○, activity; □—□, pH curve.

the molar ratio of each amino acid was calculated on the assumption that the content of arginine was 18 moles per mole of AcE, the total number of amino acid residue became 708, and the molecular weight of the protein portion was calculated to be 76,500. AcE protein contained much more acidic amino acid residues than basic ones and also more hydroxyl and carboxyl groups of amino acid residues. Titrations of the sulfhydryl group in AcE were performed with PCMB at a specifically set concentration (4.12×10^{-8} mole) and a varying concentration of PCMB in the presence of 8 M urea in 10 ml of 0.1 M phosphate buffer at pH 7.0 at 25°C. The changes in absorbance of the reaction mixtures at 250 nm, corrected for the absorbance of PCMB in the same solvent in the absence of AcE, were measured at time intervals. The reaction for 16 hr or more gave the maximum

FIG. 6. Optical Rotatory Dispersion Spectrum of AcE in Phosphate Buffer, pH 7.0.

Enzyme concentration, 2.48 mg per milliliter, light path, 0.1 mm; full scale, 0.2° .

value, 4 moles per mole AcE, as shown in Fig. 7. After performic acid oxidation, four moles of cysteic acid were detected by amino acid analysis. Thus, the enzyme was assumed to contain 4 moles of cysteine which can react with PCMB per mole of the enzyme. The estimation of extinction at 280 nm from the tyrosine, phenylalanine and tryptophan con-

FIG. 7. Spectrophotometric Titration of 8M Urea Denatured AcE with PCMB.

tents was carried out according to the method of Hsiu, Fisher and Stein¹⁷⁾ yielding a value of 24.7, which was slightly higher than the experimentally obtained 23.24. Ultraviolet absorption spectrum of AcE is shown in Fig. 8.

FIG. 8. Ultraviolet Absorption Spectrum of Crystalline AcE.

The protein (0.0996 mg) was dissolved in 1 ml of 0.1 M acetate buffer at pH 5.5. The absorption spectrum was recorded on a Shimadzu recording spectrophotometer model UV 200.

4. N-terminal and C-terminal analyses

The reaction with 2-fluorodinitrobenzene was performed in the presence of 0.1 M sodium bicarbonate at 40°C . The reaction product was successively washed with HCl, acetone and ether. The dinitrophenyl (DNP-) protein (about 10 mg) was hydrolyzed in an evacuated sealed tube with 6 N HCl at 110°C for 12 hr. DNP-amino acid was extracted with ether from the hydrolyzate and chromatographed on silica gel thin-layer plate. Two-dimensional chromatography was performed with a toluene-pyridine-ethylenchlorohydrin-0.8 N NH_4OH (100: 30: 60: 60, v/v) system in the first direction and a chloroform-methanol-glacial acetic acid (95: 5: 1, v/v) system in the second direction. N-terminal analysis showed that the N-terminal of the enzyme was threonine. No other DNP-amino acid was detected.

For the confirmation of DNP-threonine, hydrolysis by ammonium hydroxide was applied to the DNP-amino acid fraction and the regenerated amino acid was also identified as threonine by amino acid analysis.

Determination of N-terminal amino acid of AcE by applying bacterial aminopeptidase gave the same result. After 120 min of reaction with 10 nmoles of AcE, 9 nmoles of threonine was first split off, then 4 nmoles of serine and valine and 4 nmoles of phenylalanine, in that order. From these results, threonine was concluded to be the N-terminal amino acid of AcE protein.

The C-terminal amino acid of AcE was liberated by carboxypeptidase A and Y. After 2.5 hr of reaction, 5 nmoles of serine, 3 nmoles of tyrosine and 3 nmoles of aspartic acid were liberated by 0.5 units of carboxypeptidase A from 30 nmoles of AcE protein, but the liberation of serine did not proceed very rapidly. In order to ascertain the C-terminal amino acid of AcE, the rates of release of amino acids by carboxypeptidase Y were followed quantitatively. The results are summarized in Fig. 9. For the first 20 min, five amino acids were liberated rapidly; *i.e.*, serine and tyrosine appeared, accompanied by further release of aspartic acid, threonine and glycine. The control experiments which contained only carboxypeptidase Y yielded no detectable amino acids. The C-terminal amino acid of AcE protein was concluded to be serine.

5. Detection of carbohydrate

Total content of carbohydrate in the enzyme was found to be 3.34 percent by the phenol-sulfuric acid method and also by the anthrone method using glucose as a standard. The molar content in AcE protein molecule was calculated to be 16 moles being assumed as glucose. No glucosamine and galactosamine was detected on the amino acid analyzer. Three or more components other than glucose in the carbohydrate moiety were detected on TLC. The carbohydrates in AcE protein will be investigated in further experiments.

FIG. 9. Time-Course of the Release of Amino Acids from AcE Protein by Carboxypeptidase Y.

AcE protein, 3.75 mg, was dissolved in 200 μ l of 0.1 M pyridine-acetate buffer at pH 5.5. Carboxypeptidase Y, 15 μ l, containing about 15 μ g was added to the reaction mixture. After 0, 20, 40, 80, 160 and 320 min, each 20 μ l of sample was withdrawn and mixed with 20 μ l of 10% acetic acid. After being dried under N_2 gas flow, the material was dissolved in 50 μ l of citrate buffer, pH 2.2, and applied to an amino acid analyzer after the removal of any insoluble material by centrifugation.

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