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Enzymatic properties of a ferulic acid esterase from *Aspergillus sojae*

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Abstract

A major ferulic acid esterase（EC 3.1.1.73）was purified from a solid culture of *Aspergillus sojae* as an electrophoretically homogeneous protein. The molecular mass of the purified enzyme was estimated to be 30 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 29 kDa by gel filtration chromatography. The isoelectric point of the enzyme was 3.8. The purified enzyme had the highest activity toward partial hydrolyzate of starch free wheat bran with Driserase（HSFWB），and comparative but lower activities against methyl ferulate, methyl *ρ*-coumarate, methyl sinapinate and methyl 3,4-dimethoxy cinnamate. The findings therefore suggested that purified enzyme classified as type C or type D ferulic acid esterase.

Key words : *Aspergillus sojae,* Ferulic acid esterase, Ferulic acid, Starch free wheat bran

INTRODUCTION

The ferulic acid（4-hydroxy-3-methoxycinnamic acid）is not only widely used as a cosmetic material and a health food material but also is closely related to the flavor of the brewed food.[1] In our research, it has been clarified that the generation of 4-ethylguaiacol（4-EG），that is a characterized flavor of soy sauce, is discussed *Candida versatilis* converts the ferulic acid into 4- EG.[2] The ferulic acid in soy sauce mash is released from wheat bran by ferulic acid esterase of koji mold, so the enzyme is closely related to the flavor of soy sauce. In soy sauce manufacture, *Aspergillus sojae* is commonly used with *A. oryzae* for producing of *koji*, since *A. sojae* is closely related to *A. oryzae* taxonomically. Although the ferulic acid esterse from *A. oryzae* is already reported,[3, 4] little is known of molecular properties of that from *A. sojae*. In a previous paper, we purified a ferulic acid esterase from *A.sojae* for first time.[5] Here, we describe the enzymatic properties of the ferulic acid esterase, and compare its properties with those of the known enzyme of *Aspergillus* spp.

MATERIALS AND METHODS

Aspergillus sojae No.3（ATCC 200440）was used in this study. The strain was cultured on potato dextrose agar at 30 ℃ and the stock culture was kept in a refrigerator at 4 ℃. The strain was cultured in a 500-ml Erlenmeyer flask containing 20 g of wheat bran, 0.4 g of urea and 12 ml of tap water at 27 ℃ for 48 h. Ferulic acid esterase（EC 3.1.1.73）was purified as an electrophoretically homogeneous protein from the solid culture of *Aspergillus sojae*. Endo-1,4-β-D-xylanase（Xnase X-I, EC 3.2.1.8）and α-L-arabinofuranosidase（AFdase, EC 3.2.1.55）from the strain were purified according to the method of Kimura *et al.*[6]

Starch-free wheat bran（SFWB）was prepared according to the method of MacKenzie *et al.*,[7] and the ferulic acid content of SFWB was measured by the method of Faulds and Willianson.[8] The partial hydrolyzate of SFWB with Driserase （Sigma-Aldrich Japan K.K. Tokyo）:（HSFWB）and *O*- [5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose（FAXX）were prepared according to the method of Borneman *et al.*[9] The methyl derivatives of hydroxycinnamic acids and hydroxybenzoic acids were prepared according to the method of Bornenman *et al.*[10] Ferulic acid was purchased from Sigma-Aldrich K.K.. 2-Ethyl-5-phenylisoxazolium-3-sulfonate（2E5P）and *ρ*-bromophenacyl bromide（PBPB）were purchased from Tokyo Chemical Industry Co., Ltd（Tokyo）. Other chemicals

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were obtained from Wako Pure Chemical Industries（Osaka）.

Enzyme activity was measured on the basis of the release of ferulic acid from HSFWB. Unless otherwise specified, an assay mixture containing 0.4 ml of 0.125% HSFWB solution in 0.05 M 3-morpholinopropanesulfonic acid（MOPS）buffer（pH 6.5）and 0.1 ml of appropriately diluted enzyme solution was incubated at 40 ℃ for 10 min. The reaction was terminated by adding 0.75 ml of methanol, and ferulic acid released was determined with a HITACHI LaChrom high-performance liquid chromatography（HPLC）system using RSpack DE-431 column（Showa Denko, Tokyo; 4.6 x 150 mm）at 310 nm. Elution was conducted with 0.03 M phosphoric acid: methanol（4 : 6, v/v）as mobile phase at flow rate 0.8 ml/min and at 40 ℃. One unit of enzyme activity was defined as the amount of enzyme which liberated 1μmol of ferulic acid per minute under described assay conditions. Native polyacrylamide gel electrophoresis（native-PAGE）was performed according to the method of Davis.[11] Sodium dodecyl sulfate（SDS）-PAGE was performed according to the method of Laemmli.[12] The protein in the gel was stained by Coomassie brilliant blue. Gel isoelectoric forcusing was done on a thin-layer gel（Bio-Rad Labs., Richmond, CA, USA）. The method used for p*I* determination was based on the procedure reported by Låås *et al*..[13] The molecular weight of the purified enzyme was determined by gel filtration chromatography（GFC）using TSK gel G3000SW$_{XL}$（Tosoh Co., Tokyo）and SDS-PAGE. The protein content of the enzyme was measured according to the method of Bradford[14] with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The solid culture（0.5 kg）was soaked in distilled water（1.0 *l*）at 4 ℃ for 5 h, and then filtered through 4 layers of cheese cloth. The filtrate was fractionated by ammonium sulfate precipitation（40–90% saturation）, and dialyzed against 0.05 M sodium phosphate buffer（pH 6.5）. The enzyme solution was loaded onto a DEAE-Sepharose FF（GE Healthcare Japan Corporation, Tokyo）column（5.0 x 27 cm）, which was equilibrated with 0.05 M sodium phosphate buffer（pH 6.5）. After washing the column with the buffer, the enzyme in the column was eluted with a linear gradient of NaCl from 0 to 0.5 M in the buffer at a rate of 60 ml/h. Two peaks exhibiting enzyme activity（F-1 and F-2）were obtained, but the major fraction of the enzyme activity（33%）was localized in the F-2 fraction. The major fraction（F-2）was dialyzed against the buffer containing 40% saturated ammonium sulfate, and after that the fraction was loaded onto a Phenyl-Sepharose 6 FF column（GE, 2.5 x 35 cm）equilibrated with the same buffer containing 40% saturated ammonium sulfate. The column was washed first with equilibrated buffer and then eluted with a linear gradient from 40 to 0% saturated ammonium sulfate of the same buffer at rate of 10 ml/h. The active fraction was concentrated by ultrafiltration（UK-10; Advantec Toyo Co. Ltd., Tokyo）, loaded onto a Sephacryl S-100 HR column（GE, 2.5 x 95 cm）equilibrated with 0.05 M sodium phosphate buffer（pH 6.5）containing 0.1 M NaCl, and eluted with the buffer at a rate of 10 ml/h. The active fraction was dialyzed against 0.01M sodium phosphate buffer（pH 6.5）and stored in an ice bath.

Table 1 shows a summary of the steps used to purify the ferulic acid esterase F-2. The purified enzyme had a specific activity 27.5 units per mg protein, and recovery of the activity was about 19% based on the crude enzyme preparation. The purified enzyme showed a single protein band after native-PAGE. The molecular mass of native enzyme was estimated to be 29 kDa by GFC. On SDS-PAGE gel, the purified enzyme gave a single protein band, and the molecular mass of was estimated to be 30 kDa（Fig. 1）. The specific activity and molecular mass of the purified enzyme（F-2）showed the same value as a previous report[5] except that an active fraction was

Table 1. Summary of purification of ferulic acid esterase from *A. sojae*.

Step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Crude extract	870	356	339.3	1.0	100
$(NH_4)_2SO_4$ precipitation (40–90% saturation)	168	336	166.3	2.0	94
DEAE-Sepharose-FF					
F-1	120	11	12.0	0.9	3
F-2	152	119	27.4	4.3	33
F-2 purification					
Phenyl-Sepharose6FF	55	107	3.9	27.4	30
Sephacryl S-100HR	14	66	2.4	27.5	19

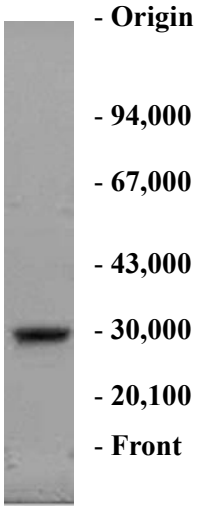

Fig. 1. SDS-polyacrylamide gel electrophoresis of ferulic acid esterase.
Purified enzyme (10 μg) and LMW standard proteins (GE Healthcare Life Science). were treated with SDS at 100 ℃ for 2 min, after which electrophoresis was carried out at 10 mA for 2 h with 0.1% SDS.

divided into two fraction. The isoelectric point of the purified enzyme was estimated to be 3.6 by thin-layer polyacrylamide gel isoelectrophoretic analysis.

The optimum pH and temperature for the activity of the purified enzyme using HSFWB as a substrate were pH 6.5 and 50 ℃, respectively. The purified enzyme was stable at 4 ℃ for 6 days in the pH range 5.5–9.0. The activity was stable up to 40 ℃ at pH 6.5, and completely lost after incubation at 65 ℃ for 15 min without HSFWB. Purified enzyme solution (0.08 units) was preincubated in a mixture containing various chemicals at 20 ℃ for 30 min and residual activities were then measured under the standard assay conditions. Addition of Cu^{2+} (1 mM), Ni^{2+} (1 mM), and *ρ*-chloromercuribenzoate (0.1 mM) showed weak inhibitory effect (less than 15%). In contrast, phenylmethylsulfonylfluoride (PMSF, 1 mM) inhibited the activity completely, while 2E5P and PBPB (1 mM each) exhibited 15% inhibitory effect on the enzyme activity. These results suggested that serine, histidine and aspartic acid residues are present in the active site residues of the enzyme. Table 2 shows the substrate specificity of the purified enzyme toward various substrates. The purified enzyme had the highest activity toward HSFWB, and comparative but lower activities against methyl ferulate (MFA), methyl *ρ*-coumarate (M*ρ*CA), methyl sinapinate (MSA) and methyl 3,4-dimethoxy cinnamate (MdMCA). The enzyme showed some activity toward methyl caffeate (MCA), however it had no activity toward methyl vanillate (MVA) and methyl syringate (MsyA). The findings therefore suggested that purified enzyme classified as type C or type D ferulic acid esterase (FEase, EC 3.1.1.73), [15] those act on all four hydroxycinnamic acid methyl esters.

Table 2. Substrate specificity of ferulic acid esterase from *A. sojae*.

Substrate[*1]	Relativeactivity (%)
HSFWB	100
MFA	72
MρCA	54
MSA	48
MCA	27
MdMCA	46
MVA	n.d.[*2]
MsyA	n.d.

[*1] HSFWB, hydrolyzate of starch-free wheat bran with Driselase [9]; MFA, methyl ferulate; M*ρ*CA, methyl *ρ*-coumarate; MSA, methyl sinapinate; MCA, methyl caffeate; MdMCA, methyl 3,4-dimethoxy cinnamate; MVA, methyl vanillate; MsyA, methyl syringate. [*2]n.d., not detected. The enzyme was reacted with various substarates in a mixture containing 0.2 units of purified enzyme, and 1.0 mg of HD-SWB or 1.0 mg of synthetic substrates in 0.5 ml of 0.05 M MOPS buffer (pH 6.0) at 40 ℃ for 10 min. The activity of various substrates was measured by described method in the text with ferulic acid as a standard.

In Table 3, some properties of FEase from *Aspergillus* spp. were compared. Although *A.sojae* is distinguished from *A. awamori* and *A. niger* taxonomically, the molecular characteristics of their FEase were similar. However, the enzyme purified from *A. sojae* differs from the FEases of the other aspergilli in terms of its substrate specificities of methyl derivatives of hydroxycinnamic acids. Although FEase has already been purified various *Aspergillus* spp., [3, 4, 16–18] the details of properties and molecular characteristics of the FEase from *A. sojae* are reported here for the first time.

For release of ferulic acid from SFWB, 0.2 units of the FEase with or without 0.6 units of Xnase X-I or AFdase was mixed with 10 mg SFWB in 1 ml of 0.05 M MOPS buffer (pH 6.5). The mixture was incubated at 30 ℃ for 98 h. At definite times, aliquots (0.05 ml) of the reaction mixture were collected, and the released ferulic acid content was determined by HPLC. The enzyme mixture of FEase, Xnase X-I and AFdase released 35.2% of ferulic acid from SFWB in the reaction mixture, the value was *ca.* 5 times higher than that without Xnase X-I in the reaction mixture. On the other hand, AFdase had little effect in releasing ferulic acid from SFWB. Addition of enzyme (0.2 units each) at 72 h did not induce the liberation of ferulic acid from SFWB (Fig. 2). Accordingly, the Xnase X-I had a synergy effect for releasing of ferulic acid

Table 3. Properties of ferulic acid esterase from various *Aspergillus* spp.

Properties	*A. sojae* No. 3[*1]	*A. oryzae* [3]	*A. oryzae* [4]		*A. awamori* [16]	*A. niger* [17]
			AoFaeB[*2]	AoFaeC[*2]		FAE-III
Molecular weight:						
SDS-PAGE	30 kDa[*3]	30 kDa[*3]	61,000	75,000	35,000	36,000
GFC	29 kDa[*3]	19 kDa[*3]	—	—	—	14,500
Isoelectoric point (p*I*)	3.6	3.6	—	—	3.8	3.3
Optimum pH	6.5	4.5–6.0	6.0–7.0	6.0–7.0	5.0	5.0
Optimum temperature (℃)	50	—	45	55–60	45	—
pH stability	5.5–9.0[*4]	3.0–7.0[*5]	3.0–9.0[*6]	7.0–10.0[*6]	4–11[*7]	—
Thermal stability (℃)	up to 40	up to 45	up to 55	up to 55	up to 50	—
Relative activities on						
MFA	100	—	2	4	—	43
MρCA	75	—	100	41	—	0.04
MCA	38	—	10	100	—	n.d.
MSA	67	—	n.d.	0.2	—	100
Inhibitor[*8]	PMSF (1 mM)	—	—	—	PMSF (10 mM)	—
					DIFP (1 mM)	—
K_m value toward FAXX (mM)	0.034	—	—	—	—	0.019 [18]

[*1]In this study. [*2]Recombinant enzyme. [*3]Molecular mass. [*4]Incubated at 4 ℃ for 6 days. [*5]Incubated at 45 ℃ for 24 h. [*6]Incubated at room temperature for 1 h. [*7]Data not shown. [*8]Indicates fully inhibition. PMSF, phenylmethylsulfonylfluoride. DIFP, diisopropylfluorophoshate. GFC was performed on a G3000SW$_{XL}$ column (Tosoh) equilibrated with 0.05 M sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl at 10 ℃. —, not determined. n.d., not detected.

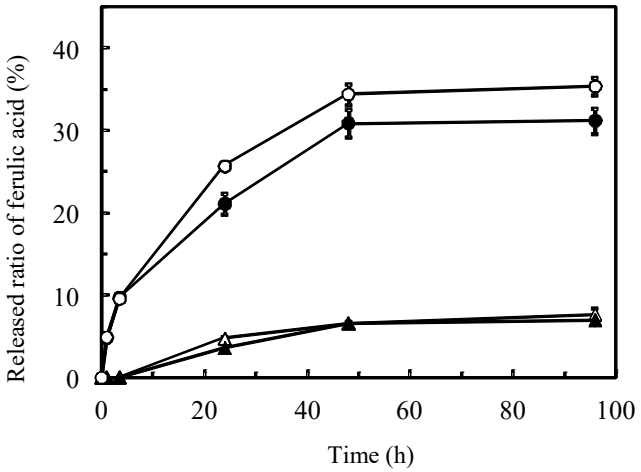

Fig. 2. Release of ferulic acid from starch free wheat bran (SFWB) by the various enzyme reactions.
The reaction mixture contained 10 mg of SFWB and 0.2 units of the enzyme or 0.2 units of each enzyme mixture in 1 ml of 0.05 M HEPES buffer (pH 6.5), and was incubated at 40 ℃. At intervals, the ferulic acid liberated was measured by HPLC (in the text). 100% of the ferulic acid was defined as the total amount of alkali-extractable acid (3.1 μg/mg SFWB). Each value is presented as mean ± SE of 5 samples. The arrow shows the enzyme added point into the reaction mixture.
Symbols: ▲, ferulic acid esterase; △, ferulic acid esterase with AFase; ●, ferulic acid esterase with Xnase X-I; ○, ferulic acid esterase with Xnase X-I and AFdase.

from SFWB, because the enzyme did not act on releasing ferulic acid from it.

The Xnase X-I from *A. sojae* has 5 subsites, and the catalytic site of the enzyme is located between third and fourth subsite counting from terminal site, which is attached to the non-reducing end of the substrate. [19] So, we considered that the FAXX might be a major product from SFWB by the Xnase X-I. Under the hypothesis, we analyzed the enzyme reaction parameter of the FEase. Hanes-Woolf plots showed the apparent K_m and V_{max} values of the FEase on FAXX were 0.034 mM and 14 μmol/min/mg protein, respectively. The K_m value of the enzyme was almost similar for that of *A. niger* FAE-III (0.019 mM), whereas remarkabl difference was observed for V_{max} value (114 μmol/min/mg protein) for FAXX of the strain. [18] These findings suggest that the FEase of *A. sojae* is closely correspoding to the production of ferulic acid during the soy sauce manufacturing process, and the xylanase operates synergistic effect for releasing the ferulic aicd. Moreover, the molecular activity of FEase from *A. sojae* was different from that of *A. niger* clearly.

We are now studying the kinetic properties of the FEase, and trying the cloning of the enzyme gene from *A. sojae* to clarify the mode of liberation of ferulic acid from wheat bran.

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*Aspergillus sojae*のフェルラ酸エステラーゼの精製と諸性質の検討

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要　約

フェルラ酸エステラーゼ（EC 3.1.1.73）は*Aspergillus sojae*の固体培養物から電気泳動的に均一に精製された．精製酵素標品の分子質量はドデシル硫酸ナリウムポリアクリルアミドゲル電気泳動で30kDa，ゲル濾過クロマトグラフィーで29kDaと見積もられた．等電点は3.8であった．精製酵素標品はドリセラーゼ処理した脱澱粉小麦ふすま部分加水分解物（HSFWB）に対して最大活性を示したが，フェルラ酸メチル，ρ-クマル酸メチル，シナピン酸メチル及び３，４−ジメトキシシナミン酸メチルにも弱いながら作用した．これらの結果から，精製酵素標品は，フェルラ酸エステラーゼのCあるいはDタイプに分類されることが示唆された．