Partial Purification and Some Properties of An Alkaline Arylsulfatase Produced by *Aspergillus* Fungi

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Arylsulfatases reacting in alkaline condition have been detected in many *Aspergillus* fungi grown on defatted soybean meal or wheat bran. The activity was produced being associated with fungal growth but the activity seemed to be unstable under the optimum incubation temperature for growth. The arylsulfatase (arylsulfatase III) active in the pH range from 7 to 10 with an optimum pH of 8.5 has been partially purified from the solid culture of *A. sojae* SH 10-6 grown on defatted soybean meal. Arylsulfatase III was unstable above 20°C, stable in the pH range from 8 to 10, and inhibited by Ag⁺, Zn²⁺, Hg²⁺, fluoride, borate, and PCMB, but not by cyanide, molybdate, and tungstate. Another fraction obtained from *A. awamori* R-0827, which was purified with the same manner as the case of *A. sojae* SH 10-6, showed closely similar enzymatic properties to arylsulfatase III of *A. sojae* SH 10-6. These enzymatic properties are clearly different from those of arylsulfatase I and II previously noted.

Arylsulfatases (EC 3.1.6.1) have been detected in various fungal strains,¹⁾ and might be present as multiple forms. The arylsulfatases produced by *Aspergillus* fungi were active in acid condition.^{2~5)} In a previous paper,⁶⁾ some properties of two arylsulfatases (arylsulfatase I and II) active in acid condition were described, and some evidence for the presence of an arylsulfatase reacting in alkaline condition was also shown. Such an arylsulfatase has not been reported in the cultures of *Aspergillus* fungi, and the activity reacting in alkaline condition was only detectable for the cultures of *Aspergillus* fungi by *koji* method (solid culture on cereal materials), but scarcely for the submerged culture.

The present paper shows that *A. oryzae*, *A. sojae* and some *Aspergillus* strains cultivated by *koji* method produce an arylsulfatase reacting in alkaline condition. Partial purification and some enzymatic properties of this arylsulfatase from *A. sojae* or *A. awamori* grown on defatted soybean meal medium are also described.

MATERIALS AND METHODS

Organisms and culture. As shown in Table I, 18 strains of *Aspergillus* maintained in our laboratory were used in this study. These strains were streaked on *koji* extract agar slant and incubated at 30°C for several days to prepare the seed culture. Five grams of wheat bran (above 2 mm in diameter) or defatted soybean meal (commercial product, 2 to 8 mm in diameter) was taken in a 200 ml Erlenmeyer flask, moistened with 4 ml of tap water, and sterilized at 120°C for 15 min. For larger scale culture, 15 g of defatted soybean meal and 12 ml of tap water were taken in a transformed Roux flask (about 500 ml, tortoise-shaped), and sterilized in the same way as above.

The conidia from the seed culture were inoculated to the medium mentioned above, and incubated under the condition described in the text.

Crude extract of arylsulfatase. Each strain grown on the defatted soybean meal or wheat bran was dispersed in 10 volumes of distilled water, mixed well, and stood with occasional shaking in a cold room (about 4°C) for 2 hr. Then the mixture was filtered, and the filtrate was used as the crude enzyme extract.

Arylsulfatase assay. Arylsulfatase activity was given by measuring the amount of *p*-nitrophenol liberated from *p*-nitrophenyl sulfate. Reaction mixture was composed of 0.5 ml of 6 mM *p*-nitrophenyl sulfate, 1 ml of buffer solution (0.2 M Tris-acetate buffer for pH 7 to 9, and 0.2 M diethanolamine-HCl buffer for

pH 8.5 to 10) and 0.5 ml of enzyme solution. The reaction was proceeded routinely for 30 min at 20°C, and was terminated by the addition of 3 ml of 1 M Na₂CO₃. The amount of *p*-nitrophenol liberated was determined by measuring the absorbance at 400 nm with a spectrophotometer (Hitachi type 101, Hitachi Ltd., Tokyo). One unit of enzyme activity was defined as the amount of enzyme which liberated 1 μ mol of *p*-nitrophenol per min at 20°C.

Protein determination. Protein contents of the enzyme solution and the column-chromatographic eluates were determined conveniently by measuring of absorbance at 280 nm with a spectrophotometer (Hitachi type 124, Hitachi Ltd., Tokyo).

RESULTS

Formation of the arylsulfatase reacting in alkaline condition in Aspergillus fungi

Eighteen strains of *Aspergillus* fungi were inoculated in pairs on the media made from defatted soybean meal or wheat bran, and each in the pair was incubated at 25 or 35°C, respectively. As shown in Table I, the arylsulfatase activity assayed at pH 8.5 (Tris-acetate buffer) was detected in all the crude enzyme extracts obtained from the cultures

of each strain. *A. sojae* SH 10-6 seemed to be the most effective strain as the alkaline arylsulfatase producer.

A. sojae SH 10-6 and *A. awamori* R-0827 (an effective strain in black *Aspergillus* group, which is commonly called in Japanese fermentation industry) were cultured on the defatted soybean meal and wheat bran media at 25, 30, and 35°C, respectively. The arylsulfatase activity of each culture was assayed at every 24 hr interval in the prolonged incubation period. As shown in Fig. 1, on the defatted soybean meal medium both strains revealed that the higher incubation temperature resulted in the shorter lag of arylsulfatase formation and the faster arrival to a maximum activity followed by marked decrease in the activity. The level of the maximum activity at lower incubation temperature was higher than that at higher incubation temperature. As shown in Fig. 2, the two strains grown on the wheat bran medium showed similar progress of the arylsulfatase formation as on the defatted soybean meal medium. These results suggest that the

TABLE I. ARYLSULFATASE ACTIVITY IN THE CRUDE ENZYME EXTRACT OF VARIOUS *Aspergillus* STRAINS

Medium	Defatted soybean meal		Wheat bran	
Incubation temp. (°C)	25	35	25	35
Strain used				
<i>Aspergillus oryzae</i> A	25.7	7.7	27.9	47.1
<i>A. oryzae</i> KB	5.0	5.8	10.8	12.9
<i>A. oryzae</i> K	17.2	7.3	29.8	38.5
<i>A. oryzae</i> A 1-5	4.7	2.2	6.1	9.8
<i>A. oryzae</i> No. 35	14.5	10.8	16.4	23.1
<i>A. oryzae</i> S4-15	31.0	24.9	33.2	28.1
<i>A. sojae</i> SH 10-1	13.3	7.4	26.1	28.4
<i>A. sojae</i> SH 10-3	7.9	17.5	24.6	23.7
<i>A. sojae</i> SH 10-6	147	70.0	30.0	120
<i>A. sojae</i> SH 21	4.9	30.5	16.3	25.7
<i>A. awamori</i> R-0827	62.0	28.0	15.0	34.0
<i>A. awamori</i> R-3523	5.4	9.5	3.9	10.0
<i>A. usamii</i> R-1031	16.9	8.0	6.1	13.6
<i>A. usamii</i> R-1219	9.0	19.4	6.4	19.1
<i>A. inuii</i> R-3631	13.8	26.2	5.3	16.9
<i>A. inuii</i> R-5823	24.9	18.5	5.8	23.1
<i>A.saitoi</i> R-0638	1.9	1.9	1.3	3.1
<i>A. niger</i> var. <i>fermentarius</i>	7.4	17.8	1.5	5.2

The enzyme activity was assayed at pH 8.5, 20°C for 30 min, and expressed as units $\times 10^{-2}$ per flask.

FIG. 1. The Time Courses of the Arylsulfatase Formation on the Defatted Soybean Meal Medium under the Different Incubation Temperatures.

The enzyme activity was assayed at pH 8.5, 20°C for 30 min. A; *A. sojae* SH 10-6. B; *A. awamori* R-0827. The molds were cultured at 25°C —○—, 30°C —△—, and 35°C —□—.

FIG. 2. The Time Courses of the Arylsulfatase Formation on the Wheat Bran Medium under the Different Incubation Temperatures.

A; *A. sojae* SH 10-6. B; *A. awamori* R-0827. The illustration of this figure is in the same manner as Fig. 1.

alkaline arylsulfatase is produced in association with fungal growth, but the activity is

unstable at 30°C or 35°C which seems the optimum incubation temperature for fungal growth.

Partial purification of arylsulfatases reacting in alkaline condition

Purification procedures were as follows, and all the procedures were done in a cold room (about 4°C).

Step 1. Fractionation with ammonium sulfate. The crude enzyme extract was prepared from the culture of *A. sojae* SH 10-6 or *A. awamori* R-0827 grown on the defatted soybean meal medium at 30°C for 48 hr. Powdered ammonium sulfate was added to the crude enzyme extract to give 3 M concentration with constant stirring. The solution was adjusted to pH 9 by the addition of an ammonia solution. After allowing to stand for 2 hr, the resulting precipitate was collected by centrifugation (10,000×*g*, 15 min) and dissolved into a small volume of 0.02 M diethanolamine-HCl buffer (pH 9.0). Insoluble material was removed by centrifugation.

The supernatant solution in Visking tube was dialysed against 50 volumes of 0.02 M diethanolamine-HCl buffer (pH 9.0) for 24 hr.

About 85% of the total activity in the crude enzyme extract was present in the enzyme preparation, and significant change in the pH-activity profile were not recognized between the crude enzyme extract and the resultant solution for both strains.

Step 2. DEAE-cellulose column chromatography. Each solution obtained at Step 1 was applied to a DEAE-cellulose column (2.1×40 cm) equilibrated previously with 0.02 M diethanolamine-HCl buffer at pH 9.0. The arylsulfatase was eluted with a linear gradient of zero to 0.5 M NaCl in the same buffer. The flow rate was maintained at 20 ml per hour, and 5 ml fractions were collected. Results are shown in Figs. 3 and 4 for the enzyme preparations obtained from the cultures of *A. sojae* SH 10-6 and *A. awamori* R-0827, respectively. The arylsulfatase activities estimated at pH 7.5 and 9 were eluted in similar positions between 0.15 and

FIG. 3. DEAE-cellulose Column Chromatography of the Step 1 Fraction Obtained from the Culture of *A. sojae* SH 10-6.

Chromatographic procedures are described in the text. Enzyme activities of eluates were assayed at pH 7.5 (—○—) and pH 9.0 (—●—). Broken line (---) shows the absorbance of eluate at 280 nm, and straight line (—) is a concentration of NaCl in the eluate. The bar in the figure shows active fractions collected.

FIG. 4. DEAE-cellulose Column Chromatography of the Step 1 Fraction Obtained from the Culture of *A. awamori* R-0827.

Chromatographic procedures are described in the text. The illustration is shown in the same manner as Fig. 3.

0.25 M of NaCl for both the enzyme preparations. Active fractions were pooled and concentrated by ultra-filtration using an UM-10 Diaflo membrane (Amicon Co., U.S.A.).

Step 3. Gel filtration on Sephadex G-200. Five milliliters of the concentrated preparation of Step 2 was applied to a Sephadex G-200 column (2.5×38 cm) equilibrated previously with 0.02 M diethanolamine-HCl buffer (pH 9.0), and eluted with the same buffer. The flow rate was maintained at 20 ml per hour, and 5 ml fractions were collected. The results of the Sephadex G-200 gel filtration are given in Figs. 5 and 6 for the preparations of *A. sojae* SH 10-6 and *A. awamori* R-0827, respectively. The active fractions were eluted between fraction No. 21 and 32, and the peak

FIG. 5. Sephadex G-200 Gel-filtration of the Step 2 Fraction Obtained from the Culture of *A. sojae* SH 10-6.

Gel-filtration procedures are described in the text. The illustration is shown in the same manner as Fig. 3.

FIG. 6. Sephadex G-200 Gel-filtration of the Step 2 Fraction Obtained from the Culture of *A. awamori* R-0827.

Gel-filtration procedures are described in the text. The illustration is shown in the same manner as Fig. 3.

was seen in fraction No. 26 for either preparation of *A. sojae* SH 10-6 or *A. awamori* R-0827.

Table II shows a summary of the purification procedure of the arylsulfatases from the cultures of *A. sojae* SH 10-6 and *A. awamori* R-0827 grown on the defatted soybean meal medium. The arylsulfatases obtained from *A. sojae* SH 10-6 and *A. awamori* R-0827 were purified 60-fold and 45-fold with yields of 40% and 60%, respectively.

Some properties of the partially purified arylsulfatase reacting in alkaline condition

Optimum pH and pH stability. The activities of the two enzyme preparations were assayed at various pH values. As shown in Fig. 7, the pH-activity profiles of the two enzyme preparations were closely similar to each other. Both the preparations were practically active between pH 7 and pH 10, and pH optima were seen at about pH 8.5.

TABLE II. A SUMMARY OF THE PURIFICATION PROCEDURE OF THE ARYLSULFATASES

Strain	<i>A. sojae</i> SH 10-6			<i>A. awamori</i> R-0827		
	Total activity ^a (A)	Absorbance at 280 nm (B)	Specific activity (A/B)	Total activity ^a (A)	Absorbance at 280 nm (B)	Specific activity (A/B)
Fraction						
Crude enzyme extract	1574	10087	0.157	274	9850	0.028
Salting out precipitate	1336	1600	0.835	234	1513	0.155
Dialysate	1278	975	1.31	230	1371	0.168
DEAE-cellulose fraction	895	229	3.91	196	376	0.521
Gel-filtration fraction	638	67.2	9.49	167	131	1.27
Yield	40.5%			60.9%		
Purification rate ^b	60.5			45.6		

^a Enzyme activity is expressed as units $\times 10^{-2}$.

^b Purification rate is shown by the ratio of the specific activity of gel-filtration fraction to that of crude enzyme extract. The enzyme activity was assayed at pH 8.5, 20°C for 30 min.

FIG. 7. pH-activity Profiles of Partially Purified Arylsulfatases.

A, *A. sojae* SH 10-6; B, *A. awamori* R-0827.

The pH of each aliquot of the two enzyme preparations was adjusted to indicated pH value with buffer (pH 4 to 6, 0.05 M acetate buffer; pH 6 to 8.5, 0.05 M Tris-acetate buffer; pH 8.5 to 10, 0.05 M diethanolamine-HCl buffer), and incubated at 20°C for 30 min. After the incubation, each aliquot was cooled rapidly in ice-cold water. The pH of cooled aliquot was readjusted to pH 8.5 with 0.2 M diethanolamine-HCl buffer. Figure 8 shows the curves of residual activity plotted against the incubation pH of the enzyme preparation. The enzyme activities of the two preparations were stable in the range between pH 8 and 10 and were inactivated rapidly below pH 6. Significant difference in pH stability was not seen between the two purified arylsulfatases.

Thermal stability. Both the enzyme solutions were adjusted to pH 8.5 with 0.05 M diethanolamine-HCl buffer and incubated at various temperatures. At every interval of 20 min during incubation, a definite volume

FIG. 8. pH-stability of Partially Purified Arylsulfatases.

Experimental method is described in the text. The curves show relative residual activity. —, *A. sojae* SH 10-6; ---, *A. awamori* R-0827.

FIG. 9. Thermal Stability of Partially Purified Arylsulfatases under Various Incubation Temperatures.

Experimental method is described in the text. The curves show relative residual activity. Incubation temperature: —○—, 20°C; —●—, 25°C; —△—, 30°C; —▲—, 40°C. Strain: —, *A. sojae* SH 10-6; ---, *A. awamori* R-0827.

of the enzyme solution was drawn and cooled rapidly in ice-cold water, and remained activity was estimated at 20°C for 30 min. The results are shown in Fig. 9 for the enzyme solutions of *A. sojae* SH 10-6 and *A. awamori* R-0827. The activities of both enzyme solutions were sustained fully at 20°C for 1 hr, but prolonged incubation period or higher incubation temperature caused gradual or apparent decrease in activity. The enzyme solution from *A. awamori* R-0827 seemed to be somewhat more stable than that of *A. sojae* SH 10-6.

Influence of metal ions. For the evaluation of the influence of metal ions, the enzyme reaction was done at pH 8.5 for 30 min at 20°C with the reaction mixture containing

TABLE III. THE INFLUENCES OF METAL IONS ON THE ACTIVITIES OF THE PARTIALLY PURIFIED ARYLSULFATASES

Strain	Relative activity (%)	
	<i>A. sojae</i> SH 10-6	<i>A. awamori</i> R-0827
Metal ions		
Absence	100	100
Li ₂ SO ₄	99	98
NaCl	100	100
KCl	100	100
CuCl ₂	94	95
AgNO ₃	2	3
MgSO ₄	99	97
CaCl ₂	100	99
SrCl ₂	104	105
ZnSO ₄	16	16
CdCl ₂	61	73
HgCl ₂	21	17
AlCl ₃	66	70
MnCl ₂	90	91
FeSO ₄	76	74
CoCl ₂	95	97

The enzyme activity was assayed at pH 8.5, 20°C for 30 min in the presence of 1 mM metal ion, and then compared with the activity in the absence of metal ion.

1 mM of metal ion listed in Table III. As shown in Table III, among the metal ions tested, Ag⁺, Zn²⁺, and Hg²⁺ showed potent inhibition and Cd²⁺, Al³⁺, and Fe²⁺ also inhibited both the enzyme activities, but the

two enzymes did not require any of the tested metal ions as a cofactor for their full activity. These results indicate that a considerable similarity is present in the metal ion inhibition between the two enzyme preparations obtained from the different *Aspergillus* strains.

Influence of several enzyme inhibitors. For the estimation of the influence of several enzyme inhibitors listed in Table IV, the enzyme reaction was proceeded at pH 8.5 for 30 min at 20°C with the reaction mixture containing

TABLE IV. THE INFLUENCES OF SEVERAL ENZYME INHIBITORS ON THE ACTIVITIES OF THE PARTIALLY PURIFIED ARYLSULFATASES

Strain	Relative activity (%)	
	<i>A. sojae</i> SH 10-6	<i>A. awamori</i> R-0827
Inhibitor		
Absence	100	100
NaF	41	70
KCN	99	95
NaN ₃	95	95
(NH ₄) ₂ SO ₄	100	100
KH ₂ PO ₄	89	88
Na ₂ HAsO ₄	100	100
NaAsO ₂	98	94
Na ₂ WO ₄	97	99
Na ₂ MoO ₄	98	98
Na ₂ B ₄ O ₇	14	14
PCMB ^a	26	38
EDTA ^b	93	97

^a *p*-Chloromercuribenzoate.

^b ethylenediaminetetraacetate

The enzyme activity was assayed at pH 8.5, 20°C for 30 min in the presence of 1 mM inhibitor, and then compared with the activity in the absence of inhibitor.

1 mM of inhibitor. As shown in Table IV, fluoride, borate, and PCMB showed potent inhibitory effect on the activities of both enzymes, and phosphate inhibited weakly. Cyanide, sulfate, arsenate, arsenite, tungstate, molybdate, and EDTA did not inhibit the activity at the concentration tested. These effects of several enzyme inhibitors for the two enzymes were similar to each other and could not distinguish the difference of strains as enzyme sources.

DISCUSSION

In the previous paper,⁶⁾ two arylsulfatase fractions conveniently named as arylsulfatase I and II, which had pH optima of 5.0 and 6.7, respectively, were partially purified, and some evidence for the presence of another arylsulfatase reacting in alkaline condition (arylsulfatase III) were indicated in the crude enzyme extracts obtained from some *Aspergillus* fungi grown on wheat bran cultured by *koji* method. Such an arylsulfatase reacting in alkaline condition has not been reported on the cultures of *Aspergillus* fungi, and the activity seems to be due to an unknown enzyme.

In the present paper, we showed that the activity of arylsulfatase III was detectable in all the tested strains when cultured by *koji* method, and predominant or enduring formation of arylsulfatase III in the prolonged culture of *A. sojae* or *A. awamori* was seen at rather lower culture temperature than the optimum temperature for fungal growth. The arylsulfatase III fraction has been purified 60-fold with 40% yield from the crude enzyme extract obtained from *A. sojae* SH 10-6 grown on defatted soybean meal. The purified fraction was practically active between pH 7 and 10 with the optimum pH at about pH 8.5. The activity was stable at 20°C between pH 8 and 10 and inactivated rapidly below pH 6, but the activity was unstable above 20°C. The purified fraction was inhibited markedly by Ag^+ , Zn^{2+} , Hg^{2+} , PCMB, and borate, but did not by tungstate and molybdate. Also the arylsulfatase III fraction from *A. awamori* R-0827, which was

prepared by the parallel purification procedure with that of *A. sojae* SH 10-6, showed quite similar properties to those of *A. sojae* SH 10-6.

These results suggest that arylsulfatase III is present in common with the solid cultures of various *Aspergillus* fungi, and arylsulfatase III is produced in association with fungal growth, but the activity is unstable and difficult to be detected at the optimum temperature for fungal growth. Arylsulfatase III can be distinguished from arylsulfatase I and II in the fact that arylsulfatase III is insensitive to tungstate and molybdate, potent inhibitor for arylsulfatase I and II. In addition, arylsulfatase III is apparently differentiated from the arylsulfatase C of vertebrates⁷⁾ and algal arylsulfatase of *Chlamidomonas reinhardtii*,⁸⁾ as arylsulfatase III is not inhibited by cyanide.

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