Korean J. Appl. Microbiol. Bioeng.
Vol. 5, No. 4, 177~184 (1977).

Purification and Properties of Arylsulfatase of *Serratia marcescens*

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(Received December 13, 1977)

Serratia marcens Arylsulfatase의 정제와 성질

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ABSTRACT

Arylsulfatase catalyzes the release of SO_4^{2-} from sulfate esters of simple phenols. Arylsulfatase occurs widely in animal tissues and in microorganisms including soil bacteria. Its widespread distribution suggests that it has a rather fundamental function and environmental meaning. It has been shown previously that arylsulfatase of *Klebsiella* was purified and characterized.

A condition of arylsulfatase synthesis was tested with several strains of *Serratia*. *Serratia marcescens* could not utilize some sugars, such as xylose, rhamnose, glucosamine and arabinose but glucose and mannitol as a sole carbon source. However, arylsulfatase synthesis was repressed by glucose but not by mannitol. The enzyme synthesis was repressed ob inorganic sulfate and methionine, and this repression was relieved by addition of tyramine.

Arylsulfatase of *S. marcescens* was purified by fractionation with ammonium sulfate and followed by chromatographies on DEAE-Cellulose CM-Cellulose, and DEAE-Sephadex A-25. The molecular weight of arylsulfatase was determined to be 46,000 by SDS-Gel electrophoresis and 49,000 by Sephadex G-100 column chromatography. The enzyme showed some different properties with that of *K. aerogenes*. The activity was maximum at pH 6.8. The Km and Vmax values for *p*-nitrophenyl sulfate were 2.5×10^{-4} M and 20 nmoles/min/mg protein, respectively. The enzyme showed high activities toward phenyl sulfate, *o*- and *p*-nitro phenyl sulfates, and *p*-nitrocatechol sulfate. The inhibition of enzyme was strongly affected by hydroxylamine, inorganic fluoride, sulfide and phosphate, but by inorganic sulfate. Like *Klebsiella* arylsulfatase, tyramine, octopamine, and dopamine gave significant inhibitory effect.

INTRODUCTION

Arylsulfatase distribute widly in microorganism, in most animal tissues and even in plant seed. In view of the widespread occurence of arylsulfate esters in nature, microorganisms synthesizing this enzyme are considered by many to be important for sulfate ester mineralization in soils that contain high levels of sulfur in the form of ester sulfate.

There are number of works on arylsulfatase synthesis which was done mostly with *Aerobacter aerogenes*[1~6] and *Klebsiella aerogenes*.[7~10,23] Arylsulfatase synthesis is controlled by sulfur source in medium.[3] In *K. aerogenes*, arylsulfatase is synthesized when the organism is grown with methionine or taurine as the sulfur source (nonrepressing conditions) and is repressed by inorganic sulfate or cysteine, this repression is relieved by addition of tyramine or related compounds (derepressing conditions).[9] Arylsulfatase was purified and its properties were reported in *K. aerogenes* W70.[10] Furthermore convenient method using affinity chromatography, tyrosyl-hexamethylenediamino-β-1, 3-glucan and immunoadsorbent, was developed for purification of the *Klebsiella* enzyme.[24]

In the Enterobacteriaceae, arylsulfatase activity was found in some strains of *Klebsiella*, *Enterobacter*, *Serratia*, and *Proteus*.[25] It was also found that the strains in the family Enterobacteriaceae could be divided into two groups on the basis of the immunological properties of their enzyme. Antisera formed a precipitin band with both active and inactive enzyme proteins from *Escherichia*, *Citrobacter*, *Salmonella*, *Klebsiella*, and *Enterobacter*, but not with the proteins from *Serratia*, *Proteus*, and *Erwinia*, even though some strains at these species had enzyme activity.[25] This observation prompted me to study the *Serratia* arylsulfatase. This paper reports the purification and properties of the Arylsulfatase from *Serratia marceecens* IFO 3046.

MATERIALS AND METHODS

1. Strains

Serratia marcescens IFO 3046 was principally used. Three strains of *S. marcescens* (IFO 3735, IFO 12648, IFO 12946), *S. indica* (IFO 3759), and *S. marinorubra* (IFO 12973) were used for the test of arylsulfatase synthesis.

2. Culture conditions

The cultures were grown aerobically at 30°C in minimal medium containing 0.05 M potassium phosphate buffer (pH 7.2), 0.1% NH_4Cl, 0.01% $MgCl_2 \cdot 6H_2O$, 0.001% each of NaCl, $MnCl_2 \cdot 4H_2O$ and $FeCl_3 \cdot 6H_2O$, and 1 mM each of Na_2SO_4 and tyramine. The carbon compound used are described for each experiment. The organisms were maintained with occasional transfer on slants of 2% agar containing nutrient broth (Difco) and 0.5% NaCl and pH 7.2. Growth was followed in a Klett-Summerson Colorimeter (590~660 nm).

3. Arylsulfatase assay

Arylsufatase activity was measured photometrically using *p*-nitrophenyl sulfate (PNPS) as substrate. When whole cells were used as enzyme preparation, they were collected by filtration on Whatman glass fiber paper GF/F with aspiration and washed with 5 ml of of 0.1 M Tris (hydrpxymethyl) aminomethane, (Tris)-chloride buffer, pH 7.5. Enzyme preparation (0.5 ml) was mixed with 2.5 ml of the same buffer containing 7.5 μmole of PNPS, and the reaction mixture was incubated for 5 to 60 min at 30°C. Reactions were terminated at appropriate times by adding 0.5 ml of N NaOH containing 0.1 M Na_3PO_4.

The fiber paper and cells were removed by centrifugation, and the optical density of the supernatant was measured in a Hitachi Spectrophotometer, Type 124 at 400 nm. One unit of activity was defined as the amount causing liberation of 1 μmole of *p*-nitrophenol per min. To study substrate specificity, the arylfatase activities activities towards various substrates were assayed by the method of Dodgson and Spencer,[11] with the modification that 0.2 ml of the enzyme preparation in a centrifuge tube was mixed with 0.5 ml of substrate solution in 0.1 M Tris-chloride buffer, pH 7.2.

4. Tests on inhibition by chemical compounds

The enzyme activity was assayed using 20 μg of purified enzyme per ml. After neutralization of aqueous solution of the compounds by adding hydrochloric acid, they were added to the enzyme solution in Tris-chloride buffer pH 7.2, prior to initiation of the reaction by addition of PNPS.

5. Protein concentrations

Protein concentration were determined by the method of Lowry *et al.*[12] with bovine serum

albumin as a standard.

6. Electrophoretical analysis

Polyacrylamide gel electrophoresis was carried out by the method of Davis.[13] The concentration of polyacrylamide was 7.5% and each column was run at 5 mA in Tris glycine buffer, pH 8.3. Coomassie Brilliant Blue (0.2%) in methanol-acetic acid-H_2O (5:1:5, v/v) was used for staining protein bands and the pigment in free form was removed by soaking the gel in 7% acetic acid-5% methanol solution.

7. Molecular weight

Molecular weight was determined by SDS-gel electrophoresis and by Sephadex G-100 column chromatography, following the methods of Weber and Osborn,[14] and Whitaker,[15] respectively.

8. Chemicals

Tyrosine-*o*-sulfate and tyramine-*o*-sulfate were synthesized by the method of Dodgson *et al.*[16] Phenyl sulfate, *o*-, *m*- and *p*- nitrophenyl sulfate and *o*-, *m*- and *p*-carboxyphenyl sulfate and phenolphthalein disulfate by a modification of the method of Whitehead *et al.*[17] *p*- Nitrocatechol sulfate and indoxyl sulfate were obtained from Sigma Chemicals Co. Other chemicals were of the purest grade available commercially.

RESULTS

1. Arylsulfatase synthesis in various strains of Serratia

Table 1 shows growth and the activities of arylsulfatase in various strains of *Serratia* in minimal medium with and without tyramine, with methionine or inorganic sulfate as the sole sulfur source. Arylsulfatase activity shows the highest value in cells of *Serratia marcescens* IFO 3046 grown with tyramine and Na_2SO_4. The enzyme synthesis was repressed when cells were grown in inorganic sulfate and methionine medium in the absence of tyramine. Addition of tyramine to the same medium caused the synthesis of arylsulfatase. It had been reported previously that inorganic sulfate caused repression of arylsulfatase synthesis and that this repression was derepressed by tyramine.[3,6] However, in the strains of *Serratia* methionine also repressed arylsulfatase synthesis. *S. marcescens* IFO 3735 and *S. Indica* IFO 3759 synthesize arylsulfatase, but the other strains of *Serratia* could not. Therefore I used *S. marcescens* IFO 3046 for further experimences. Growth and arylsulfatase activies in *S. marcescens* IFO 3046 was also tested in medium varried with several carbon sources (Table 2). The synthesis of arylsulfatase was affected by carbon compound. Glucose affected arylsulfatase synthesis but not the growth. In *K. aerogenes*, glucose inhibits the action of tyramine in relieving repression of arylsulfatase synthesis caused by inorganic sulfate or cysteine.[8] Xylose, rhamnose, glucosamine, and L-arabinose repressed the cell growth significantly, whereas mannitol, glycerol, sorbitol, and mannose were utilized as a good carbon source, and arylsulfatase synthesis was occured.

Table 1. Growth and Arylsulfatase Activity in Several Strains of *Serratia*.

S source	Growth & activity	IGO					
		3046	3735	3759	12648	12946	12973
Na_2SO_4	Klett U.	182	211	130	250	195	178
	mU/mg Cell	0.32	0.11	0.09	0.06	0.08	0.09
Methionine	Klett U.	208	249	150	263	223	203
	mU/mg Cell	0.33	0.32	0.23	0.18	0.17	0.08
Na_2SO_4+Tyramine	Klett U.	212	208	220	270	205	250
	mU/mg Cell	6.36	1.05	1.05	0.25	0.51	0.60
Methionine+Tyramine	Klett U.	115	220	160	260	215	225
	mU/mg Cell	6.25	1.04	1.71	0.45	0.16	0.55

Table 2. Effect of Carbon Source on Cell Growth and Arylsulfatase Activity in *Serratia marcescens*.

Carbon source	Growth (doubling time)	Activity
Glucose	90 (min)	4.3 (mU/mg cell)
Fructose	120	8.4
Mannose	120	7.6
Sorbitol	150	10.5
Rhamnose	>1000	—
Glycerol	120	9.0
Mannitol	90	8.6
Xylose	>1000	—
Glucosamine	300	—
L-Arabinose	>1000	—
Succinate	150	9.0
Citrate	180	11.7

2. Purification of arylsulfatase

S. marcescens was grown in mannitol-sulfate medium containing tyramine. The cells in stationary growth phase were harvested by centrifugation from the cultures in 20 liters. All operations were performed at 4°C.

Step 1. The cells were washed with 0.1 M Tris-HCl buffer, pH 7.2, suspended in the same buffer and then disrupted by sonication for 20 min at 20'KC in a Ultrasonic Oscillator (Kaijo-Denki, Type 4280) in ice water bath. The cell debris was removed by centrifugation at 10,000×g for 20 min and 2,700 ml of supernatant, containing about 260 munits of arylsulfatase, were obtained as crude enzyme.

Step 2. Solid ammonium sulfate was added to the crude enzyme with stirring to give a final saturation of 45%. The precipitate formed was removed by centrifugation for 20 min at 15,000×g and the supernatant was brought to 65% saturation of ammonium sulfate by further addition of the salt. The resulting precipitate was collected by centrifugation and dissolved in 270 ml of 0.02M Tris-chloride buffer, pH 7.2. This solution was dialyzed against the same buffer with changes of two or three times.

Step 3. The dialyzed material was applied to a column (4.5×15 cm) of DEAE-Cellulose equilibrated with 0.02M Tris-HCl buffer, pH 7.2. The elution was performed with same buffer containing 0.1 M NaCl. Because arylsulfatase of *S. marcescens* was bound weakly that it was sometimes easy to elute when column was washed with a buffer solution. The solution (300 ml) containing arylsulfatase activity was precipitated with ammonium sulfate (70% saturation), dissolved (60 ml) in 0.02M Tris-HCl buffer, pH 7.2, and dialyzed against the same buffer.

Step 4. The dialyzed enzyme solution was applied to the column (5×20 cm) of CM-Cellulose equilibrated with 0.02M Tris-HCl buffer pH 7.2. The column was washed withed with same buffer containing M NaCl, and eluted with a linear concentration gradient of 0.05M to 0.5 M potassium phosphate buffer (300 ml each). The fraction with activity (40 ml) eluted from the column with about 0.2M potassium phosphate buffer were combined. Arylsulfatase activity emerged as a single synmetrical peak coinciding with that of protein, as shown in Fig. 1. Fractions Nos. 47 to 55 were combined and concentrated by using collodion bag.

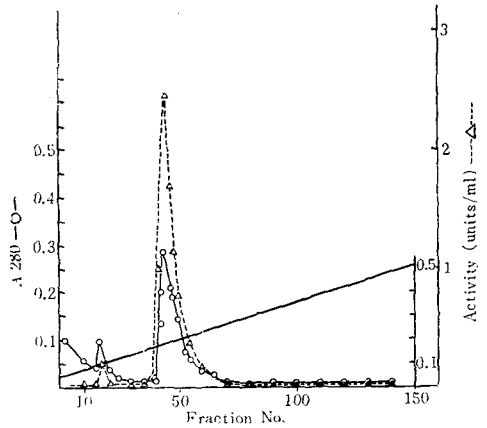

Fig. 1. Chromatography of Arylsulfatase on CM-cellulose. Concentrated and dialyzed the DEAE-Cellulose fraction was applied onto the CM-Cellulose column (5×20 cm) equilibrated with 0.02M Tris-HCl buffer (pH 7.2). Wash and elution were described in the text. Fraction Nos. 47 to 55 were combined.

Table 3. Enzyme Purification.

Step	Volume (ml)	Total protein (mg)	Total activity (munit)	Specific activity (munit)	Recovery (%)
1. Crude enzyme	2,700	12,960	259	0.02	100
2. $(NH_4)_2SO_4$ 45~65%	270	2,589	207	0.08	80
3. DEAE-Cellulose	52	125	150	1.2	58
4. CM-Cellulose	6	5.3	85	16	32
5. DEAE-Sephadex A-25	24	2.2	55	25	21

Fig. 2. Polyacrzlamide disc Gel Electrophoresis of Purified Arylsulfatase of *S. marcescens*.

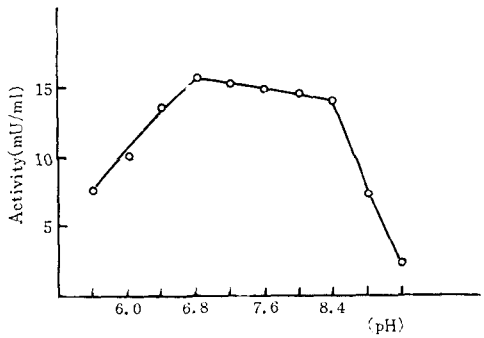

Fig. 3. Optimum pH of Arylsulfatase.

Step 5. The concentrated enzyme was applied to a column (2.2×21 cm) of DEAE-Sephadex A-25 equilibrated with 0.02M Tris-HCl buffer, containing 0.01M NaCl. Elution was performed with a linear gradient of 0.01M to 0.2M NaCl. The purification procedure is summarized in Table 3. The arylsul-

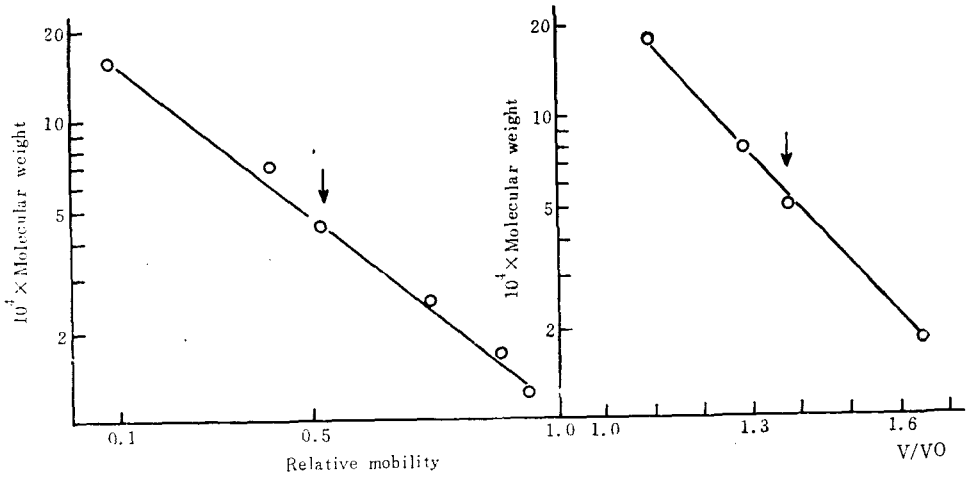

Fig. 4. Estimation of the Molecular Weight of the Arylsulfatase by SDS-gel Electrophoresis and DEAE-Sephadex G-100 Column Chromatography. The following were used as marker proteins (molecular weight).

1. Cytochrome C (12,400)
2. Myoglobin (16,900)
3. Chymotosypsinogen (25,000)
4. Ovalbumin (45,000)
5. Bovine albumin (70,000)
6. γ-Globulin (156,000)

fatase of *S. marcescens* purified about 1,250 hold (specific activity 25 munits/mg protein) with a yield of about 2.2 mg of enzyme from 20 liters of culture.

3. Purities and molecular weight of preparations of aryls ulfatase

Fig. 2 shows purified enzyme protein band on disc-gel electrophoresis at pH 8.3. The molecular weights of the enzyme was determined to be 46,000 by SDS-gel electro-phoresis and 49,000 by Sephadex G-100 column chromatography (Fig. 4). This shows almost same as that of *K. aerogenes*.[10]

The enzyme activity was amximal at pH 6.8 at 40°C for 30 min incubation (Fig. 3).

4. Kinetics of arylsulfatase

Fig. 5 shows that the Km and Vmax values of the enzyme for *p*-nitrophenyl sulfate were 2.5×10^{-4} M 20 nmoles/min/mg protein, respectively. Km and Vmax values for *p*-nitrophenyl sulfate was very lower than that of *K. aerogenes*

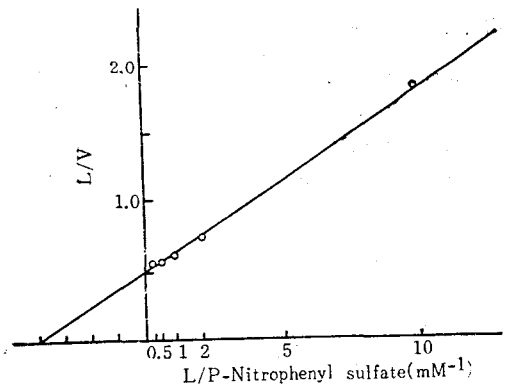

Fig. 5. Km and Vmax values of Arylsulfatase of *S. marcescens* for *p*-Nitrophenyl sulfate as Substrate. V. represents reaction velocity (nanomoles/mg of protein per minute).

5. Substrate specificity

The enzyme showed high activities towards *p*-, *o*- nitrophenyl sulfate, phenyl sulfate, and *p*-nitrocatechol sulfate. A little activities towards *m*-nitrophenyl sulfate. *o*-carboxyphenyl sulfate, indoxyl sulfate and threonine-*o*-sulfate. The enzyme did not react towards *m*-, *p*-carboxyphenyl, tyrosine-*o*-sulfate, tyramine, tyramine-*o*-sulfate and phenolphthaleine disulfate (Table 4).

Table 4. Substrate Specificity of Arylsulfatase Activity.

Substrate	SO_4^{2-} liberation μg/min/mg protein
Phenyl sulfate	10.4
o-Nitrophenyl sulfate	5.8
m-Nitrophenyl sulfate	0.4
p-Nitrophenyl sulfate	35.7
p-Nitrocatechol sulfate	9.0
o-Carboxyphenyl sulfate	0.4
m-Carboxyphenyl sulfate	0
p-Carboxyphenyl sulfate	0
Tyrosine-*o*-sulfate	0.2
Tyramine-*o*-sulfate	0.2
Indoxyl sulfate	0.4
Phenolphthaleine disulfate	0
Threonine-*o*-sulfate	0.4

Table 5. Inhibition by Inorganic Compounds.

Addition	Relative activity		
	0 mM	2.5 mM	25 mM
None	100	—	—
Na_2SO_4		108	103
Na_2SO_3		34	18
Na_3PO_4		70	32
NaF		18	9.4
NH_2OH		0	0
KCN		84	27.8

6. Inhibition of the enzyme

Table 5 shows inhibitory effects of inorganic sulfite, phosphate, fluoride, hydroxyl amine and cyanide. Hydroxylamine was inhibitory completely. However sodium sulfate did not inhibite the enzyme activity. In other hand, sodium sulfite and sodium phosphate caused about 80% and 70% inhibition, respectively. Cyanide also inhibited the enzyme activity but cyanide inhibited it completely.[10] The effects of several amino compounds on the enzyme action were investigated (Table 6). Tyramine, octopamine and dopamine gave significant inhibitory effect, but no effects were observed with tryptamine. It has been shown that tyramine and a number of phenols enhance the activity of the arylsulfatase of

Aspergillus oryzae and their effects were found to be due to phenol sulphotransferase activity caused by the arylsulfatase itself. These results are of great importance and interest because this inhibition is highly specific and the enzyme synthesis is also induced specifically by these compound.

Table 6. Inhibition by Amine Compounds.

Addition	Relative activity		
	0 mM	2.5 mM	25 mM
None	100	—	—
Tyramine		19	5
Dopamine		59	28
Octopamine		55	31
Tryptamine		81	—

DISCUSSION

It has been reported that in some microorganisms different arylsulfatase are synthesized by a single strain. A strain of *Pseudomonas aeruginosa* produces two different arylsulfatases when grown in a medium containing an arylsulfate ester as sulfur source.[19,20] The presence of multiple electrophoretic species of arylsulfatase in sonicated preparation of *Proteus rettgeri*,[21] has been demonstrated by disc electrophoresis. Two distinct fractions of arylsulfatase of *Aspergillus uidulans*, having different Km values for *p*-nitrophenyl sulfate and nitrocatechol sulfate, were separated by DEAE-Cellulose chromatography.[22] Fowler and Rammler[5] purified the enzyme of *A. aerogenes* ATCC 9621 from the cells grown in a medium containing methionine as the sulfur source, and found that it was homogeneous in molecular size. H. Okamura *et al.*[10] demonstrated that the preparations of arylsulfatase synthesized under non-repressing conditions was identified with the enzyme synthesized under derepressing conditions in *K. aerogenes* W 70. Arylsulfatase is known to occur in some strains of the family Enterobacteriaceae. However the levels of arylsulfatase activity ia these strains were found to show considerable variations that might be due to differences in enzyme structure or in the mechanisms of regulation of the enzyme synthesis. Many lines of evidence at the molecular level suggest that enteric species are related to *Escherichia coli* in the order *Shigella*, *Salmonella*, *Klebsiella* (*Aerobacter*), *Serratia, and Proteus*. This order is based on studies of the immunological cross-reactions of their enzymes, the homologies of their total deoxyribonucleic acid and ribonucleic acid, the electrophoretic mobilities of their enzymes, and interspecific genetic recombinations. Yamada *et al.*[25] found that there are significant immunological differences between the arylsulfatase protein in *Escherichia*, *Citrobacter*, *Salmonella*, *Klebsiella*, *and Enterobacter* on the other hand, and *Serratia*, *Proteus*, *and Erwinia* on the other, irrespective of whether they had enzyme activity.

In this work, I showed that arylsulfatase from *S. marcescens* was some differences in Km, Vmax, substrate specificities, and electrophoretical mobility from that from *K. aerogenes*. These results support that antiserum prepared against *K. aerogenes* arylsulfatase did not cross-react with the enzyme from *S. marcescens* and *K. areogenes* was also seen in the pattern of regulation of arylsulfatase synthesis, such as catabolite repression and methionine repression.

요 약

Arylsulfatase는 간단한 phenols류의 황에스테르 화합물로부터 SO_4^{2-} 이온의 유리를 촉매한다.

이 효소는 토양세균을 포함한 많은 미생물과 동식물의 조직등에 널리 분포하여 있으며 이와 같은 넓은 분포는 이 효소의 기본적 기능이 환경학적으로 매우 중요한 의미를 같는다고 하겠다.

Arylsulfatase에 대한 보고는 *Klebsiella* sp를 사용하여 몇몇 보고가 있다. 본 연구는 6종의 *Serratia* sp를 사용하여 arylsulfatase 합성조건을 검토하고 효소의 정제조건과 성질에 대하여 조사하여 *Serratia marcescens*를 선정하였다. *Serratia mrcescens*는 탄소원으로서 xylose rhamnose, glucosamine 그리고 arabinose등과 같은 몇몇 당을 이용하지 못했으며 glucose와 mannitol을 잘 이용 하였으나 glucose methionine의 경우 효소 합성을 억제시키었다.

유황원으로서는 무기유황염과 methionine의 첨가

는 억제되었으며 tyramine의 첨가에 의해서 효소 합성의 억제효과는 해제되었다.

효소의 정제는 황산암모늄 포화용액의 분획과 DEAE-Cellulose, CM-Cel lulose 그리고 DEAE-Sephadex A-25로 연결되는 구분 분획에 의해서 행하여졌다. 효소의 분자량은 SDS-gelelectrophoresis와 Sephadex G-100 column chromatography에 의하여 각각 46,000과 49,000으로 측정되었고 최적 pH는 6.8이었다.

P-Nitrophenyl sulfate를 사용한 Km과 Vmax치는 각각 2.5×10^{-4}M과 20 nmoles/min/mg protein이었다.

기질에 대한 특성은 phenylsulfte와 *o*-, *p*-nitrophenyl sulfate 그리고 *p*-nitro catechol sulfate에 대해서 높은 활성을 보였다.

Hydroxylamine, inorganic fluoride, sulfide 그리고 phosphate 등은 강한 효소 저해작용을 나타내었고 무기유산염은 저해작용을 보여주지 않았다.

Tyramine, octopamine 그리고 dopamine과 같은 amino acid 또한 강한 저해 작용을 보였다.

ACKNOWLEDGEMENTS

I am much oblized to professor Harada and also wish to thanks to Dr. Murooka for his valuable advices and discussion.

REFERENCES

1) Harada.: *Bull. Agr. Chem. Soc., Japan*, **21**, 267 (1957).
2) Harada T. and K. Kono: *Nippon Nogeikagaku Kaishi*, **28**, 608 (1954).
3) Harada T. and B. Spencer: *Biochem. J.*, **93**, 373 (1964).
4) Rammler, D.H. C. Crado and L. R. Fowler: *Biochemistry*, **3**, 224 (1964).
5) Fowleo L.R. and D.H. Rammler: *Biochemistry*, **3**, 230 (1964).
6) Adachi, T. Y. Murooka and T. Harada: *J. Bacteriol.*, **116**, 19 (1973).
7) Adachi, T. Y. Murooka and T. Harda: *J. Bacteriol.*, **121**, 29 (1975).
8) Adachi, T. H. Okamura, Y. Murooka and T. Harada: *J. Bacteriol.* **120**, 880 (1974).
9) Okamura, H. Y. Murooka and T. Harada. *J. Bacteriol.*, **129**, 59 (1977).
10) Okamura, H. T. Yamada, Y. Murooka and T. Harada: *Agr. Biol. Chem.*, **40**, 2071 (1976).
11) Dodgson K.S. and B. Spencer: *Biochem. J.*, **55**, 436 (1953).
12) Lowry, O.H. W.J. Rosebrough, A. L. Farr and R. J. Randall: *J. Biol. Chem.*, **193**, 265 (1951).
13) Davis. B.J. *Ann. N.Y. Acad. Soc.*, **121**, 404 (1964).
14) Weber K. and M. Osborn: *J. Biol. Chem.*, **244**, 4406 (1964).
15) Whitaker J.R.: *Anal. Chem.*, **35**, 1950 (1963).
16) Dodgson, K.S. F.A. Rose and N. Tudball: *Biochem. J.*, **71**, 10 (1959).
17) Whitehead, J.E.M. A.R. Morrison and L. Young. *Biochem. J.*, **51**, 585 (1952).
18) Burns G.R. and C.H. Wynn. *Biochem. J.*, **149**, 679 (1975).
19) Harada T.: *Biochem. Biophys. Acta*, **81**, 193 (1964).
20) Delisle G.J. and F.H. Milazzo: *Can J. Microbiol.*, **18**, 561 (1972).
21) Fitzgerald J.W. and F.H. Milazzo. *Can. J. Microbiol.*, **16**, 1109 (1970).
22) Apte B.N. and O. Siddigi. *Biochim. Biophys. Acta*, **242**, 129 (1971).
23) Murooka, Y. T. Adachi,. Okamura and T. Harada: *J. Bacteriol.*, **130**, 74 (1977).
24) Murooka, Y. M.H. Yim, T. Yamada and T. Harada: *Biochim. Biophys. Acta*, in press.
25) Yamada, T. Y. Murooka and T. Harada: *J. Bacteriol.*, in press.