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Purification of Aspartate Aminotransferase in the Liver of *Tilapia zillii*

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Abstracts

Partial purification of GOT isozymes from *T. zillii* were performed by mean of CM-cellulose column chromatography. Purified s-GOT and m-GOT fractions showed remains of a small amount of impurities on disc electrophoregrams. Zymograms of s-GOT and m-GOT fractions on starch-gel showed their specific polarities. s-GOT fraction yielded four bands on the anode side of the zymogram, whereas the m-GOT fraction yielded two bands on the cathode side. Purification of GOT of eel liver which was performed in order to compare with the GOT isozymes of tilapia liver. The GOT isozymes of eel liver had almost same characteristics as that of the tilapia liver.

Kinetic studies on s-GOT and m-GOT from liver of *T. zillii* were performed. It was recognized that the reaction of GOT isozyme is a binary mechanism, namely, s-GOT had a strong affinity for 2-oxoglutarate, whereas m-GOT had a strong affinity for aspartate.

It is well known that two isozymes of GOT exist in animal cells[1,2]. One of them is contained in the cytosol and other one exists in the matrix of mitochondria. The physiological functions of aspartate and malate are to make malate-aspartate shuttle by MDH isozymes and GOT isozymes.

Previous paper[3] has shown GOT, GDH and SDH activities in various tissues of *Tilapia zillii*. In order to more fully characterize the GOT of tilapia tissues, this paper is description of the purification and kinetic study on GOT isozymes, which are extracted and fractionated from liver of *T. zillii*.

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Abbreviations: In this paper, following abbreviations are used.

GOT : Aspartate aminotransferase (EC. 2. 6. 1. 1)
GDH : Glutamate dehydrogenase(EC. 1. 4. 1. 2)
SDH : Succinate dehydrogenase(EC. 1. 3. 99. 1)
s-GOT : Cytosol GOT
m-GOT : Mitochondrial GOT
MDH : Malate dehydrogenase(EC. 1. 1. 1. 37)
α-KG : 2-Oxoglutaric acid

Materials and Methods

Reagents : NADH (grade 3, from yeast), MDH were obtained from Sigma Chemical Co. L-Aspartic acid, 2-oxoglutaric acid, pyridoxal-5′-phosphate, sucrose, Triton X-100 and specially prepared reagents for electrophoresis were obtained from Nakarai Chemicals Ltd., Kyoto. Partially hydrolyzed starch for zymography was purchased from Electrostarch Co., Wis., U. S. A. CM-cellulose and DEAE-cellulose were obtained from Pharmacia Fine Chemicals Japan.

Animals : *Tilapia zillii*, which had a body length of 15 to 20 cm, were purchased from Kagoshima Prefecture Fisheries Experimental Station, Freshwater Branch at Ibusuki. Liver of *T. zillii* was also kindly supplied from Mr. S. Nakashima. Liver of eel, *Anguilla japonica*, was obtained from a local fish market.

Preparation of tissues and subcellular fractionation[2,4,5,6] : Preparation and subcellular fractionation were carried out by almost same methods described in previous paper[3].

Enzyme purification[7,8] : Mitochondrial and cytosol fractions were isolated from *T. zillii* liver conforming to the previously described procedure[3].

1) Purification of s-GOT

a) Heat treatment One liter of the tissue extract was added to 4 liters of maleic acid buffer (pH 6.0) which contained 0.05 M maleic acid, 0.003 M 2-oxoglutaric acid, 0.001 M EDTA and 0.0001 M pyridoxal-5′-phosphate. The mixed solution was heated to 68°C on a water bath with continuous stirring and then immediately cooled in iced water. Denatured protein was removed by centrifugation (5,000×G, 10 min).

b) Ammonium sulfate precipitation Ammonium sulfate (516 g) was added to 1 liter of the supernatant obtained by heat treatment, and after stirring for one hour at room temperature, Hyflo-super-cel was added at a concentration of 20 g per liter, and filtered with suction. The precipitate was dissolved in 0.01 M sodium acetate buffer (pH 5. 4) and filtered with suction. The filtrate was dialyzed overnight against 2 liters of 0.01 M sodium acetate buffer.

c) CM-Cellulose column chromatography After a CM-cellulose column was equilibrated with 0.01 M sodium acetate buffer (pH 5.4), the dialyzed protein was concentrated with Ultra Filtration Cell (Amicon model 52) and added on the column. Then 600 ml of 0.01 M sodium acetate buffer was passed through the column, and the enzyme fractions were eluted by a linear gradient elution method with sodium acetate buffer (pH 5. 4) from 0.04 M to 0.2 M. Flow rate was 0.7 ml per minute.

2) Purification of m-GOT

a) Heat treatment The procedure was same as described in s-GOT purification. However, the supernatant was dialyzed overnight against 2 liters of 0.1 M sodium acetate buffer (pH 5. 4).

b) CM-Cellulose column chromatography The CM-cellulose column was equilibrated with 0.1 M sodium acetate buffer (pH 5.4). After the protein solution was charged on the column, 80 ml of 0.1 M sodium acetate buffer (pH 5.4) was passed through the column. The enzyme fraction was eluted by a stepwise elution method with 0.1 M and 0.2 N sodium acetate buffer (pH 5.4).

3) Purification of GOT in eel liver Eel liver was homogenized with Ultra Turrax Homogenizer (IKA. Germany). The liver homogenate was used for purification of GOT isozymes. The procedures were almost same as the s-GOT purification method in case of *T. zillii*. But for eel liver, ammonium sulfate precipitation was performed twice. In the second treatment, ammonium sulfate was added in a concentration of 370 g per liter. An equal volume of cold acetone (−20°C) was added to the ammonium sulfate treated sample. After centrifugation at 10,000×G for 10 min, the precipitate was dialyzed overnight against 2 liter of 0.01 M sodium acetate buffer (pH 5.4). Unabsorbed fraction on the CM-cellulose column was purified by DEAE-cellulose chromatography. The DEAE-cellulose column was equilibrated with 0.005 M potassium phosphate buffer (containing 0.005 M maleic acid), and the enzyme solution was added. Then 50 ml of 0.005 M potassium phosphate buffer (pH 6.5) was added to elute the absorbed s-GOT.

Measurment of protein concentration : The protein concentration was measured by LOWRY's method[9].

Enzyme assay[1,2,11] : The enzyme activity of GOT was assayed by modified method of KARMEN[10], described in previous paper[3].

Zymography[2,12,13,14,15] : Electrophresis and staining method were same to previous paper[3].

Enzyme kinetics : The kinetic studies were performed by method of VELICK et al.[16].

Results

Purification of s-GOT from liver of *T. zillii* : The purification steps, protein concentration and GOT activities of the enzyme solution are shown in Table 1. The initial heat treatment step was performed on a 70 g of liver homogenate. In the CM-cellulose column chromatography step, the specific activity became 8 times stronger than that in the heat treatment step. The elution pattern of s-GOT on CM-cellulose column is shown in Fig. 1. GOT protein was eluted by linear gradient elution method using sodium acetate buffer (pH 5.4) in concentration of from 0.04 to 0.2 M, and two peaks of active enzyme fractions were obtained. Then they were used as sample for zymogaphy. Results of the zymography are shown in Fig. 3.

Table 1 Purification of s-GOT from *T. zillii* Liver

Purification step	Total volume (ml)	Protein (mg/ml)	Specific activity *	Recovery (%)
Heat treatment	390	2.5	3.7	100
Ammonium sulfate precipitation	81	4.6	6.3	64
CM-cellulose column	65	0.15	29.0	8

* : μ moles of product/min/mg of protein
Started by 70 g of *T. zillii* liver on wet weight.

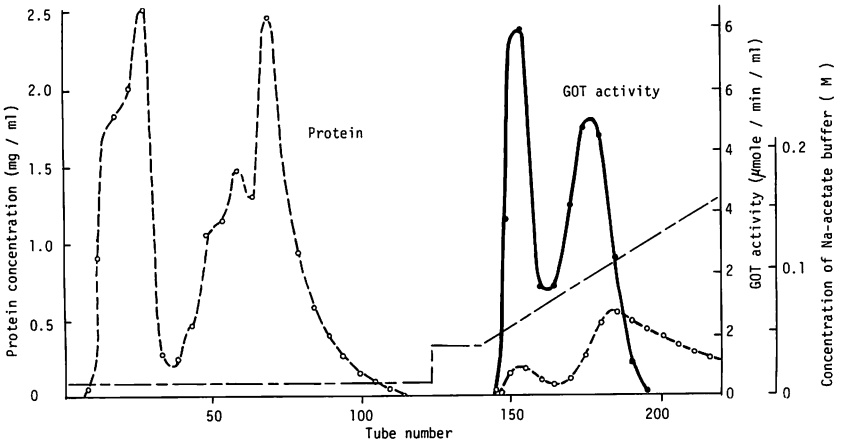

Fig. 1 CM-Cellulose Column Chromatography of s-GOT Prepared from *T. zillii* Liver
Column size: 1.8 $\phi \times 25$ cm
Flow rate : 0.7 ml/min

Purification of m-GOT from liver of *T. zillii*: Table 2 shows the purification steps, protein concentration and the enzyme activities of m-GOT prepared from liver of *T. zillii*. The heat treatment was performed on a 4 g of mitochondrial sample. The pattern resulting from CM-celluose chrmatography is shown in Fig. 2. The enzyme protein was eluted by a stepwise method with 0.2 M sodium acetate buffer (pH 5.4). Specific activity became 9 times stronger than the activity in the heat treatment step. Results of zymography of the m-GOT fractions are shown in Fig. 3.

Zymograms of s-GOT and m-GOT fractions on starch-gel show their specific

polarities. s-GOT fraction shows four bands on the anode side of the zymogram, whereas the m-GOT fraction shows two bands on the cathode side.

Table 2 Purification of m-GOT from *T. zillii* Liver

Purification step	Total volume (ml)	Protein (mg/ml)	Specific activity *	Recovery (%)
Heat treatment	17	0.64	12.1	100
CM-cellulose column	20	0.02	111.4	42

* : μ moles of product/min/mg of protein
Started by 4 g of mitochondrial fraction from *T. zillii* liver

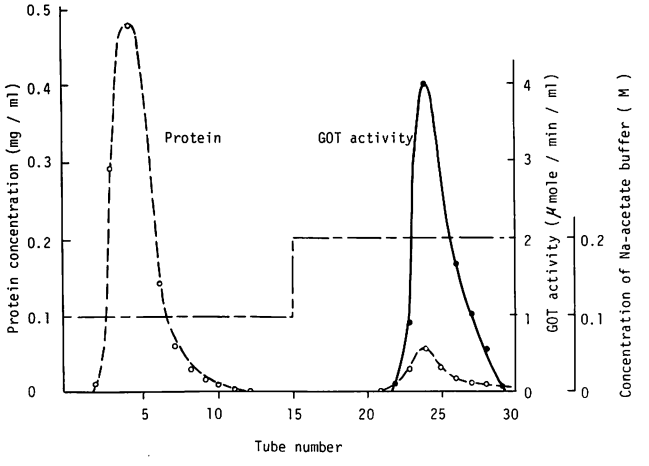

Fig. 2 CM-Cellulose Column Chromatography of m-GOT
Prepared from *T. zillii* Liver
Column size : 0.8 $\phi \times$ 15 cm
Flow rate : 0.7 ml/min

s-GOT and m-GOT from liver of eel: GOT enzyme from liver of eel, *Anguilla japonica*, was purified as control sample to the GOT of tilapia. The purification steps of the enzyme, the protein concentration and the enzyme activities are shown in Table 3. Heat treatment was performed on a 250 g of the liver homogenate. The results expressed in Fig. 4 shows two GOT active fractions. One of the GOT active fraction was not absorbed on CM-cellulose column. The other fraction was absorbed and subsequently eluted by the linear gradient method using sodium acetate buffer (pH 5.4) in concentration of from 0.01 to 0.2 M. In this step, the specific activity of GOT became

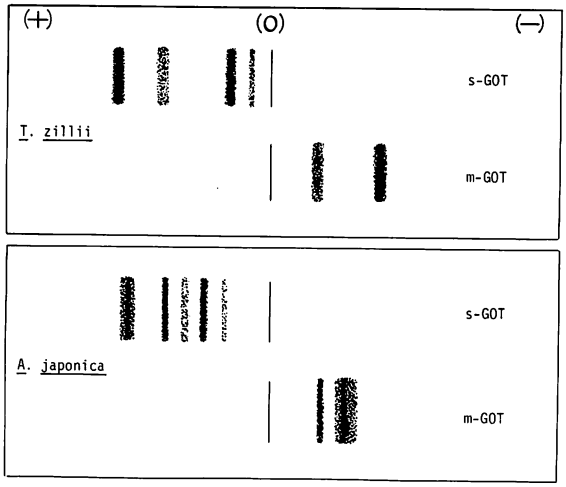

Fig. 3 Zymograms of Purified s-GOT and m-GOT Fractions Prepared from *T. zillii* and *A. japonica* Livers

Table 3 Purification of s-GOT and m-GOT Prepared from *A. japonica* Liver

Purification step	Total volume (ml)	Protein (mg/ml)	Specific activity *	Recovery (%)
Heat treatment	885	5.6	3.1	100
First ammonium sulfate precipitation	345	13.1	3.2	96
Second ammonium sulfate precipitation	127	21.0	4.7	82
Acetone treatment	101	6.8	9.6	44
CM-cellulose column	76	0.12	148.9	—
DEAE-cellulose column	18	0.09	4.7	—

Started by 250 g of *A. japonica* liver on wet weight.
* μg moles of product/min/mg of protein, at 30 °C.

about 50 times stronger than in the heat treatment step.

A protein of the unabsorbed fraction was further subjected to DEAE-cellulose column. The pattern of elution is shown in Fig. 5.

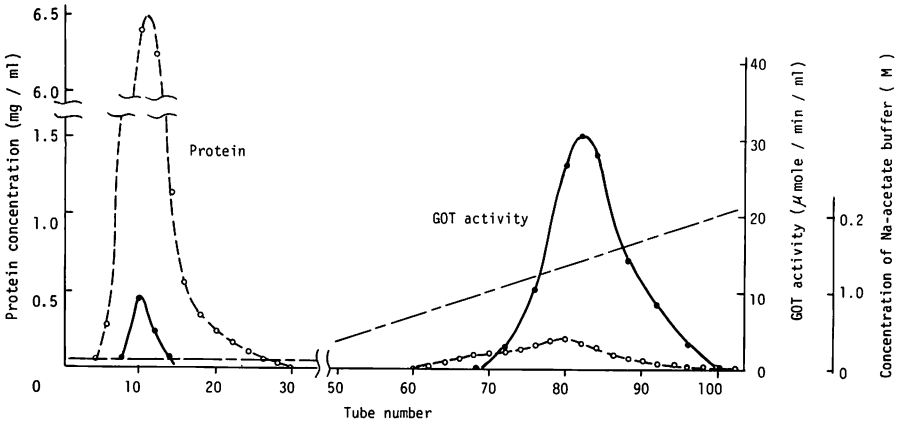

Fig. 4 CM-Cellulose Column Chromatography of GOT Prepared from Extract of *A. japonica* Liver
Column size : 1.8 $\phi \times 20$ cm
Flow rate : 0.7 ml/ min

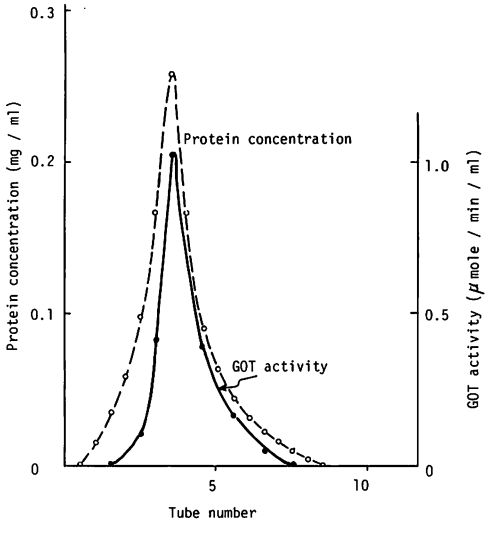

Fig. 5 DEAD-Cellulose Column Chromatography of GOT Prepared from Extract of *A. japonica* Liver
Column size : 0.8 $\phi \times 15$ cm
Flow rate : 0.7 ml/ min

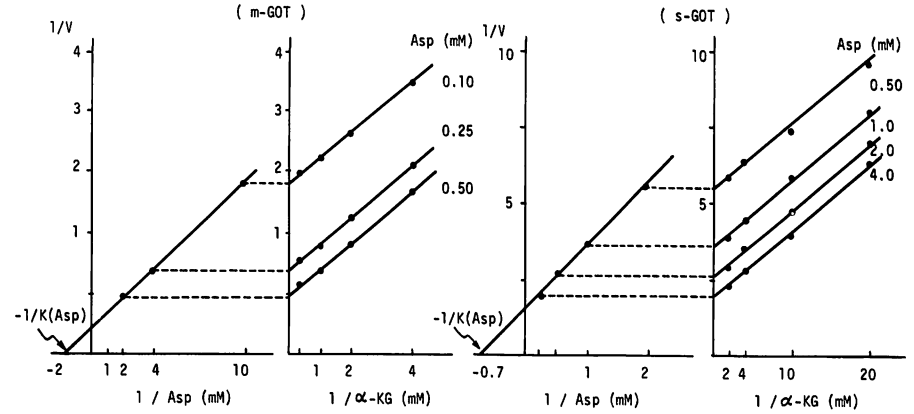

Fig. 6 LINEWEAVER-BURK Plots of Purified s-GOT and m-GOT from *T. zillii* Liver—1

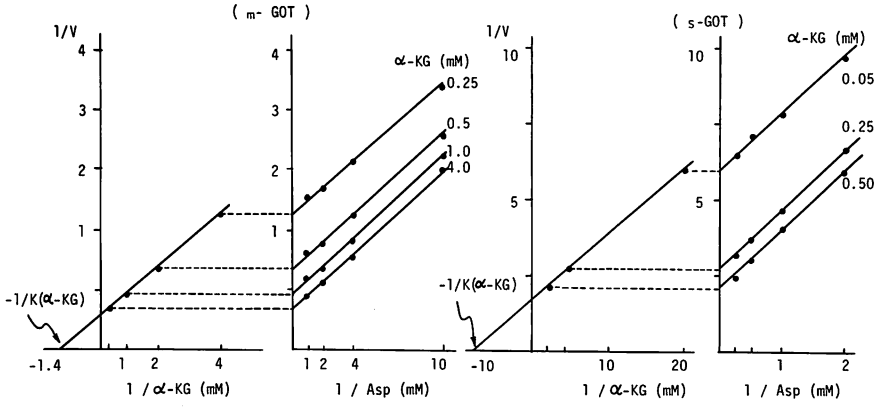

Fig. 7 LINEWEAVER-BURK Plots of Purified s-GOT and m-GOT from *T. zillii* Liver—2

Table 4 The K_m Values of GOT Enzymes

Enzyme source			K_m (mM) K_{ASP}	$K_{\alpha KG}$	Temp (℃)	pH
T. zillii	Liver	Mitochondria	0.5	0.7	30	7.5
		Cytosol	1.4	0.1	30	7.5
Eel	Liver	Mitochondria	0.3	0.8	30	7.5
		Cytosol	2.2	0.2	30	7.5
Pig[7]	Heart	Mitochondria	0.5	1.0	25	7.4
		Cytosol	2.5	0.3	25	7.4
Rat[1]	Liver	Mitochondria	0.5	1.0	25	7.4
		Cytosol	3.0	0.2	25	7.4

Starch-gel electrophoresis was then performed on these eluted fractions, and the results are shown in Fig. 3. The fraction eluted from the DEAE-cellulose column shows three distinct bands and two light bands on the anode side, thus it is identified as s-GOT. While the fraction eluted from the CM-cellulose column has two distinct bands on the cathode side, and it is identified as m-GOT.

Kinetic studies on GOT from liver of *T. zillii*: The enzyme solutions used for this experiment were s-GOT and m-GOT, which were extracted from liver of *T. zillii* and were purified on CM-cellulose column. Kinetic studies were performed by method of VELICK et al.[16]. The results are shown in the Fig. 6 and 7, and Table 4. Since each line is parallel as shown in the Figures, the results suggest that the reactions of GOT isozymes are performed on a binary mechanism. It is known that s-GOT and m-GOT have strong affinities for 2-oxoglutarate and aspartate, respectively.

Discussion

The GOT enzyme could not be purified completely in both *T. zillii* and eel livers as shown in Fig. 8. s-GOT and m-GOT of tilapia exhibited five and six protein bands on disc electrophoregrams, respectively.

GOT isozymes in cytosol and mitochondria usually contain several different molecular subforms and they are separable from each other by electrophorsis[17]. The presence of multiple forms within each isozyme was detected by starch-gel electrophoresis. From the cytosol GOT of pig heart, MARTINEZ-CARRION et al.[18] isolated no less than three main discrete protein subfractions designated as α, β and γ forms in the order of increasing anodic movility. MARINO et al.[19] achived satisfactory separation of five fractions by isoelectricfocusing. BOSSA et al.[20] detected additional subforms in pig

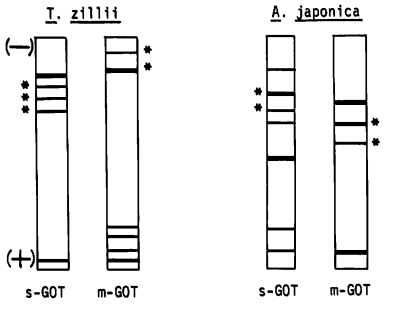

Fig. 8 Disc Electrophoresis of Purified s-GOT and m-GOT Fractions from *T. zillii* and *A. japonica*
Gel was stained by amide black 1 B in 7% acetic acid.
* had GOT activities.

heart GOT fractionated by cation exchange chromatography, and characterized them as glycoproteins with unequal carbohydrate content (up to 10%). The isozymes from tilapia in this paper were not purified completely, but, it is presumable that the four bands stained for GOT activity on anode side of the zymogram are s-GOT subforms. And also it is presumable that two GOT bands on the cathod side are m-GOT subforms.

K_m values for both aspartate and 2-oxoglutarate substrates were described in Table 4. There are differences in K_m values on these substrate reported by many investigators[1,7]. It is considered difficult to measure activity of GOT exactly. The reason is believed due to reversible reaction (in s-GOT) and substrate inhibition of 2-oxoglutarate or formed oxaloacetate (in m-GOT). Therefore, the KARMEN's method[10] employs removal of oxaloacetate from the reaction system.

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