Mem. Fac. Fish. Kagoshima Univ.,
Vol. 37, pp. 1 ~10 (1988)

Purification of Native Glutamate Dehydrogenase from Eel Liver

Seiichi Hayashi[*1], Monzoor Morshed[*1], Keiko Fukuyama[*1],
Keisuke Nakasako[*1], and Zentaro Ooshiro[*1]

Keywords : Glutamate dehydrogenase, Eel, Liver, Native GDH, Mitochondria.

Abstract

Native glutamate dehydrogenase (GDH) was purified from the eel liver through procedures including fractionation of mitochondria, absorption chromatography with hydroxyapatite, ion-exchange chromatography with DEAE-Sephacel, affinity chromatography with GTP-Sepharose, and gel filtration with Toyopearl HW-55. Molecular weight of native GDH was 340,000 and the subunit of the GDH consisted of one kind of protein with molecular weight of 53,000 to 55,000. Isoelectric point of the GDH was pH 5.9. Amino acid composition of the GDH was analyzed and had a certain similarity to that of bovine liver GDH. The composition of glycine, histidine, methionine, tryptophan, tyrosine and valine in both GDHs was very similar.

When glutamate dehydrogenase (GDH) was purified from the livers of eel (*Anguilla japonica*), two kinds of GDHs were obtained. One was native GDH and the other was GDH suffered limited proteolysis. However, at the beginning period of the study on GDH, it was not noticed that the purified GDH had suffered limited proteolysis[1-3]. We concluded at that time that the eel liver GDH consisted of two kinds of subunits with different molecular weights. Namely one was 53,000-55,000, and the other was 50,000-52,000. It was found later that the ratio of two kinds of subunits was altered due to the livers used for purification. When the livers preserved at −20℃ for several weeks were used for purification, the ratio of the protein with molecular weight of 53,000-55,000 to the protein with that of 50,00-52,000 increased. Furthermore, it was revealed that when leupeptin known as an inhibitor of trypsin or thiol proteases was used during the purification of GDH, the purified GDH consisted of only one kind of subunit of which molecular weight was 53,000-55,000. That was native GDH.

This report describes the method for the purification of the native GDH and some of its properties.

*1 Laboratory of Food Chemistry, Faculty of Fisheries, Kagoshima University, 50-20 Shimoarata 4, Kagoshima 890, Japan.

Materials and Methods

Materials

GTP-3Na was obtained from Yamasa Soybean Co. NADH was purchased from Oriental Yeast Co., Ltd. CNBr-activated Sepharose, DEAE-Sephacel, and Sephadex G-25 were purchased from Pharmacia LKB Biotechnology. TSK 4000 SW column was purchased from Toyo Soda Industry. Calibration Kits for molecular weight determination using polyacrylamide gel electrophoresis was from Pharmacia LKB Biotechnology. γ-Methylester-L-glutamate, leupeptin, and pepstatin were obtained from Peptide Institute, Inc. Methanesulfonic acid(4N) containing 0.2% 3-(2-aminoethyl) indole for amino acid analysis was purchased from Pierce Chemical Co. Ampholines for isoelectrofocusing using polyacryamide gel were from Pharmacia LKB Biotechnology.

Hydroxyapatite was prepared by the method of Tiselius *et al*[4]. GTP-Sepharose was prepared as described previously[1]. Eel livers were obtained from Sueyoshi Co. in Kagoshima. Other reagents were from Wako Pure Chemical Industries and Nakarai Chemical Ltd.

Procedures for the Purification of Native GDH

Fresh livers (100 g) in ice were transported to the laboratory and used as the starting material for purification. All steps for purification were carried out at 7℃.

(*Mitochondrial Fraction*)

Livers were homogenized in 1.26 *l* of ice-cold 0.25 M sucrose - 10 mM Tris-Cl (pH 7.5) - 0.1 mM $MgCl_2$ (0.25 M sucrose buffer) using mixer (National MX-M3) and Teflon homogenizer (20 m*l*). The homogenate was centrifuged at 1,800 rpm (500 ×g) for 10 min to remove nuclear fraction. The supernatant was centrifuged at 7,600 rpm (6,000 ×g) for 30 min and the resulting precipitate was resuspended with 0.25 M sucrose buffer. The suspension was centrifuged at 10,000 rpm (8,500 ×g) for 10 min and the resulting precipitate was resuspended in 0.25 M sucrose buffer. This procedure was repeated three times. The washed precipitate was used as mitochondrial fraction.

(*Solubilization of Mitochondrial Fraction*)

Mitochondrial fraction was solubilized by ultrasonication at 29 kHz for 20 min in 200 m*l* of 2% Triton X-100 - 0.02 M Na-phosphate buffer (pH 7.5), and centrifuged at 35,000 rpm (150,000 ×g) for 30 min. The resulting supernatant was applied to the following column to obtain purified GDH.

(*Chromatography on Hydroxyapatite, DEAE-Sephacel, GTP-Sepharose, and Toyopearl HW-55 Columns*)

The supernatant after ultrasonication was applied on a hydroxyapatite column (5.6×8cm) equilibrated with 0.02 M Na-phosphate buffer until the absorbance of the effluent at 280 nm was below 0.02, and then GDH was eluted with 0.25 M K-phosphate buffer (pH 7.5). Fractions containing GDH activity were pooled, applied on a Sephadex G-25 column (5×30cm) equilibrated with 0.05 M Tris-Cl (pH 7.5) - 1 mM EDTA, and eluted with the same buffer as the one used for the equilibration of Sephadex G-25. Fractions with GDH activity were pooled, applied to a DEAE-Sephacel column (2.4×12.4cm) equilibrated with 0.05 M

Tris -Cl (pH 7.5) - 1 mM EDTA, washed with the same buffer and eluted by a linear gradient of KCl in 0.05 M Tris-Cl (pH 7.5) - 1 mM EDTA as shown in Fig. 1. The GDH eluted from a DEAE-Sephacel column was applied to a Sephadex G-25 column (5×30cm) equilibrated with 0.05 M Tris-Cl (pH 7.5) - 1 mM EDTA to remove KCl and eluted with the same buffer. Then fractions with GDH activity were applied to a GTP-Sepharose column (2×9.5cm) equilibrated with 0.05 M Tris-Cl (pH 7.5) - 1 mM EDTA, washed with the same buffer, and eluted with a linear gradient of KCl in the same buffer. GDH was eluted as a single peak as shown in Fig. 2. The fraction with GDH activity were pooled, applied to a Toyopearl HW-55 column (1.6×86cm) equilibrated with 0.1 M NH_4HCO_3 and eluted with the same solution (Fig. 3).

Assay of GDH Activity

Assay mixture for GDH activity consisted of 0.1 M Tris-Cl (pH 8.0), 5 mM α-ketoglutarate, 100 mM NH_4Cl, 0.25 mM EDTA, and 0.15 mM NADH. GDH activity was assayed at 30℃ and the oxidation of NADH was followed by a decrease in absorbance at 340 nm with Hitachi 100-50 spectrophotometer. One enzyme unit is defined as the amount of enzyme catalyzing a decrease of one micromole of NADH per minute under the assay

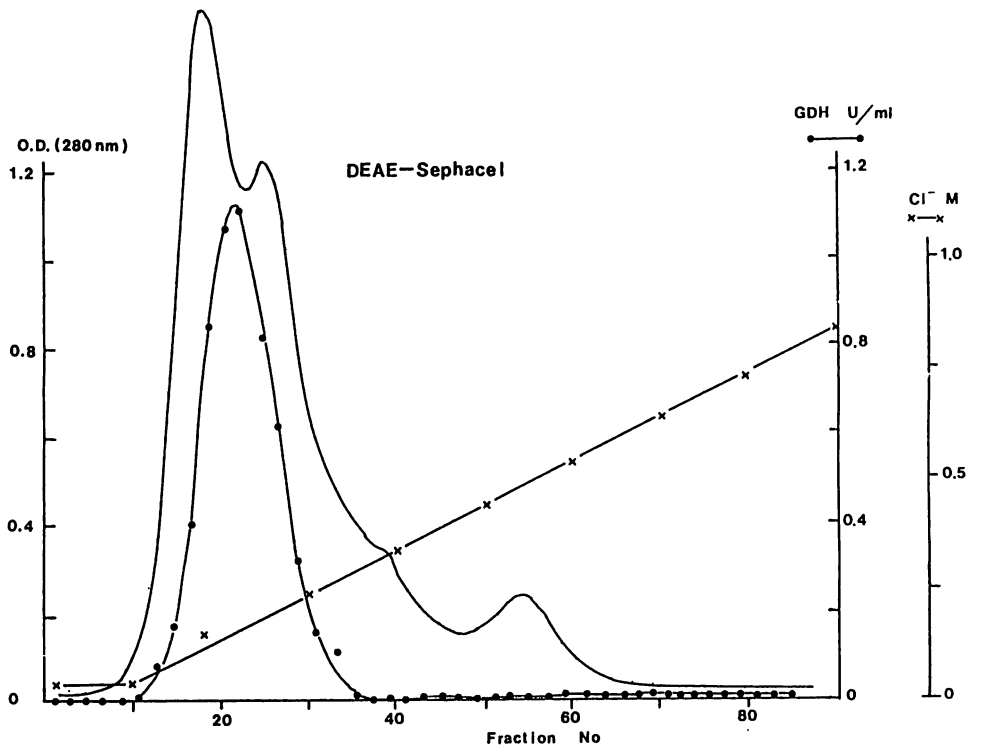

Fig. 1. Ion-exchange chromatography on a DEAE-Sephacel column. Column size, 2.4×12.4cm ; Fraction, 5 g/tube ; Buffer, 0.05 M Tris-Cl (pH 7.5)-1 mM EDTA

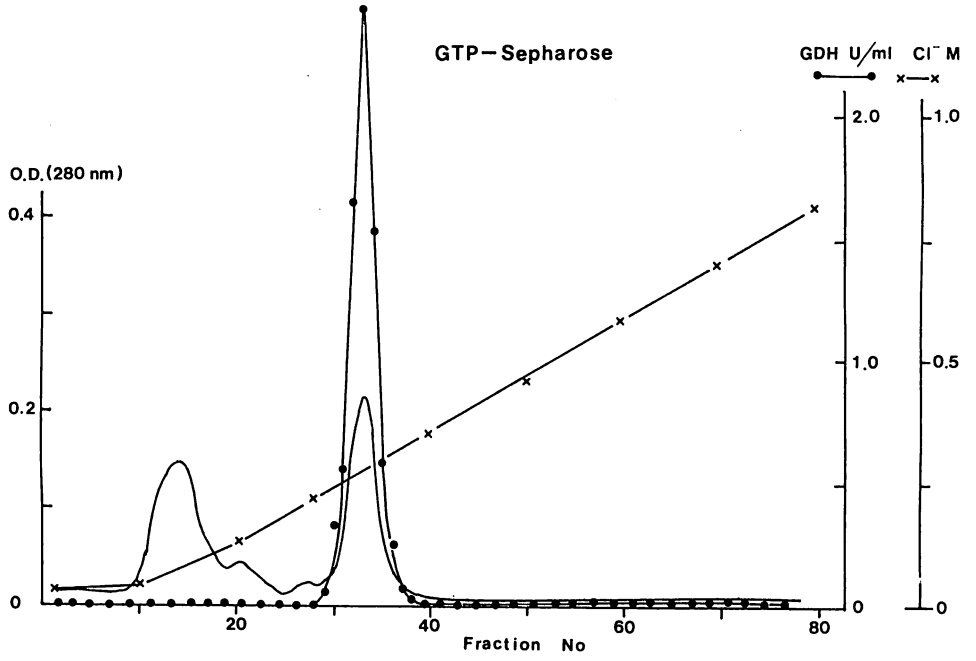

Fig. 2. Affinity chromatography on a GTP-Sepharose column. Column size, 2×9.5cm ; Fraction, 5 g /tube ; Buffer, 0.05 M Tris-Cl (pH 7.5)-1mM EDTA

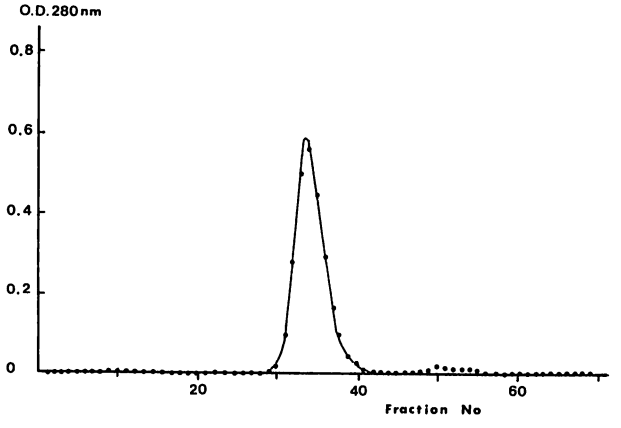

Fig. 3. Gel filtration on a Toyopearl HW-55 column. Column size, 1.6×86cm ; Fraction, 2.5 g/tube ; Eluant, 0.1 M NH_4HCO_3

condition.

Protein was assayed by the method of Bradford[5]

Determination of Molecular Weight

The molecular weight of GDH was determined by gel filtration through TSK 4000 SW column equilibrated with 0.2 M phosphate buffer (pH 6.8), using bovine liver GDH (340,000), aldolase (158,000), bovine serum albumin (68,000), ovalblumin of chicken (45,000), and chymotrypsinogen (25,000) for making calibration curve.

The molecular weight of subuint of GDH was determined by polyacrylamide gel electrophoresis in the presence of sodiumdodecyl sulfate and 2-mercaptoehtanol (SDS-PAGE). SDS-PAGE was performed according to the method of Laemmli[6].

Determination of Isoelectric Point

Isoelectric point of GDH was determined by isoelectric focusing using polyacrylamide gel electrophoresis as described by Miyazaki et al[7,8].

Sample was dialyzed against 0.1 M β-alanine, of which pI was 6.9, and 50 μl of dialyzed sample and 33 μl of 75.5% glycerol - 5% Ampholine were mixed. The mixture was applied to a gel, which consisted of 4% polyacrylamide - 5% glycerol - 2% Ampholine, and preventive solution was layered over the sample. Preventive solution consisted of 15% glycerol - 2% Ampholine - 1% glutamate-Na. Electrophoresis was performed using 0.02 M H_3PO_4 as anode solution and 1 M NaOH as cathode solution under succesive voltage of 200 V for 1 h, 300V for 5-10 h, and 400V for 1 h in the refrigerator.

After focusing was achieved, the gel was removed from the glass rods and sliced by gel-slicer to obtain gel of 3 mm size. The sliced gels were taken into test tubes, 0.5 ml of water was added and kept overnight at 4℃. The pH and GDH activity in the eluate from the gel were measured.

Analysis of Amino Acids Composition

Purified GDH was lyophilized and a precise amount of lyophilized GDH in hydrolysis tube (Pierce) was hydrolyzed in 0.5 ml of 4N-methanesulfonic acid containing 0.2% 3-(2-aminoethyl)-indole under the reduced pressure at 115℃ for 24 or 48 h. At the end of hydrolysis the equal volume of 2.5N NaOH was added in hydrolysis tube and the solution was diluted to 5 ml with water exactly. About 0.7 ml of diluted solution was used for amino acids analysis using Hitachi Amino Acid analyzer (Type 835-50).

Results and Discussion

Purification of Native GDH

Table 1 shows a typical example of the purification of native GDH from mitochondrial fraction of eel liver. The specific activity of the purified GDH was 12.7 U/mg protein, which corresponded to a 500-fold purification with respect to the homogenate. The recovery from a hydroxyapatite column was 87% as shown in Table 1 and was larger than that of prior purification step. This phenomenon occurred in every experiments of the purification of GDH. The reason which caused the increase in recovery was assumed that some inhibitors of GDH were removed by a hydroxyapatite column. The affinity chromatography by

GTP-Sepharose was available to purify GDH. The overall recovery was 48%.

So far as the mitochondrial fraction was used as a starting material, no GDH suffered limited proteolysis was obtained. Namely even though the mitochondrial fraction preserved at −20℃ for 2 to 6 weeks was used, GDH did not suffer limited proteolysis. There was no need to add leupeptin into the mitochondrial fraction. Furthermore, after the mitochondrial fraction was incubated at 25℃ for 17 h in the presence of 5 mM pyruvate, 5 mM glutamate, penicillin (70 μg/m*l*), and streptomycin (100 μg/m*l*), the GDH purified from that mitochondria was also native GDH. These results proved that the protease(s) which caused the limited proteolysis of native GDH was absent in mitochondria.

However, when GDH was purified from the supernatant and precipitate after the centrifugation of mitochondrial fraction treated with ultrasonication, both GDHs purified from the supernatant and precipitate were native, but the specific activity of the GDH purified from the precipitate was always higher than that of the GDH purified from the supernatant. The specific activity of GDH always increased when the native GDH suffered limited proteolysis. These results showed the possibility that the GDH on mitochondrial membrane suffered slight limited proteolysis of which production couldn't be recognized by SDS-PAGE.

Some Properties of Native GDH

The molecular weight of native GDH was determined to be 340,000 using a TSK 4000 SW column and the subunit consisted of only one kind of protein with molecular weight of 53,000 to 55,000 as shown in Figs. 4 and 5. The molecular size of eel liver GDH was the same as that of bovine liver GDH in gel-filtration by TSK 4000 SW. These results proved that the native GDH of eel liver was a hexamer.

The absorption maximum and minimum of the GDH in 0.05 M Tris-Cl (pH 7.5) - 1 mM EDTA was 278 and 250 nm, respectively, and the absorption coefficient at 280 nm for 1% solution of native GDH was 6.34. Isoelectric point of native GDH was pH 5.9 as shown in Fig. 6.

As shown in Table 2, amino acid composition of native GDH had a certain similarity to that of bovine liver GDH[9]. The composition of glycine, histidine, methionine, tryptophan, tyrosine, and valine in both GDHs was very similar.

Table 1. Purification of the native GDH of eel liver.

Step	Vol	Protein		GDH		Recovery
	m*l*	mg/m*l*	mg	U/mg	U	%
Homogenate	2563	9.3	23896	0.024	572	100
Mt fraction	95	24.2	2298	0.091	210	37
Ultrasonication in 2% TritonX−100	450	4.8	2160	0.063	136	24
Hydroxyapatite	155	9.1	1411	0.352	496	87
DEAE−Sephacel	140	2.4	336	0.975	328	57
GTP−Sepharose	13	1.6	21	12.7	267	47

Liver ; 200 g.

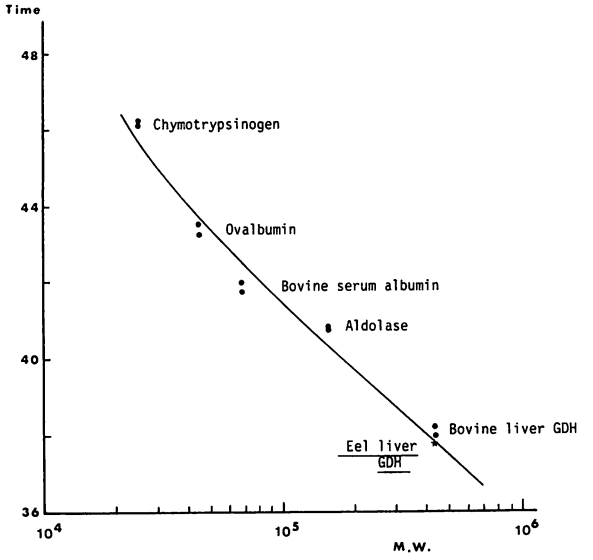

Fig. 4. Determination of molecular weight of eel liver GDH by a TSK 4000 SW column.
Standard proteings for making calibration curve were as follows ; bovine liver GDH (340,000), aldolase (158,000), bovine serum albumin (68,000), ovalbumin of chicken (45,000), and chymotrypsinogen (25,000).

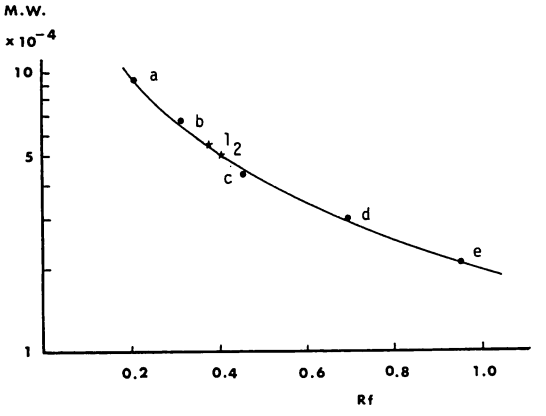

Fig. 5. Determination of molecular weight of a subunit of eel liver GDH by SDS-polyacrylamide gel electrophoresis.
a) phosphorylase b, 94,000 ; b) albumin, 67,000 ;
c) ovalbumin, 43,000 ; d) carbonicanhydrase, 30,000 ;
e) trypsin inhibitor, 20,000 ; f) α-lactalbumin, 14,4000 ;
1) native GDH of the eel liver ; 2) limited proteolyzed GDH

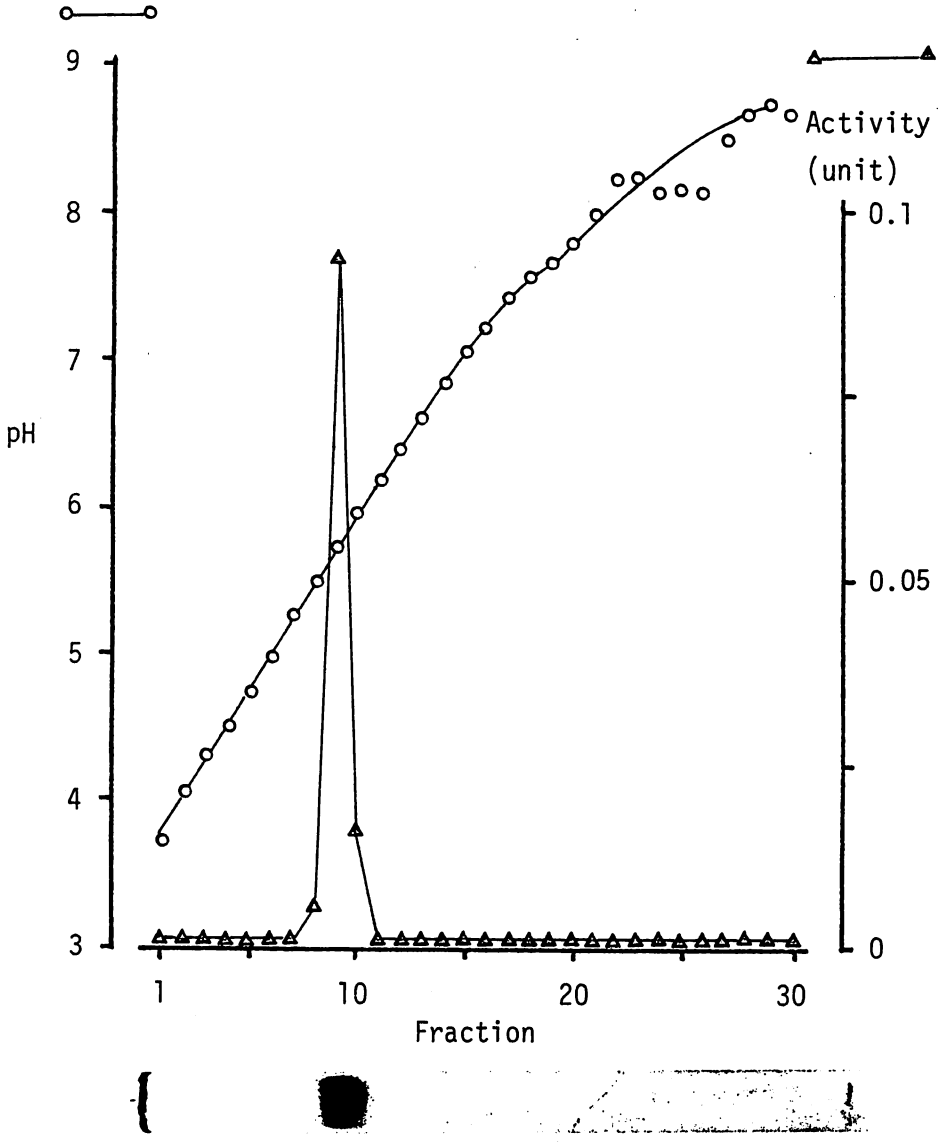

Fig. 6. Isoelectrofocusing of the native GDH purified from the eel liver.

Table 2. Amino acids composition of eel and bovine liver glutamato dehydrogenase.

	Eel Liver	Bovine Liver[9]
Ala	44	37
Arg	26	30
Asp	54	50 (Asn 21)
Glu	50	44 (Gln 13)
Gly	48	47
His	16	14
Ile	34	37
Leu	34	31
Lys	36	33
Met	12	13
Phe	20	23
Pro	26	21
Ser	20	30
Thr	22	28
Trp	2	3
Tyr	20	18
Val	36	34
Cys	–	6
Glx	–	1
	500	500

References

(1) S. Hayashi and Z. Ooshiro (1977) : Availability of GTP-Sepharose for the purification of glutamate dehydrogenase from eel liver. *Nippon Suisan Gakkaishi*, **43**, 107-115.

(2) S. Hayashi, K. Ise, T. Itakura, and Z. Ooshiro (1982) : Purification and characterization of glutamate dehydrogenase from eel liver. *Nippon Suisan Gakkaishi*, **48**, 557-562.

(3) K. Ise (1978) : Glutamate dehydrogenase of the eel liver. -Purification and enzymatic properties. (Master Thesis, The postguraduate course of Faculty of Fisheries), pp. 1-126 (in Japanese).

(4) A. Tiselius, S. Huerteu, O. Levin (1956) : Protein chromatography on calcium phosphate columns. *Arch. Biochem. Biophys.*, **65**, 132-155.

(5) M. M. Bradford (1976) : A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248-254.

(6) U. K. Laemmli (1970) : Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.

(7) K. Miyazaki, K. Hosoi, Y. Suzuki, Y. Nagao, Y. Matsuo, and T. Horio (1976) : Isoelectric fractionation. "Chemistry of protein" (ed. by N. Ui and N. Tamiya), Jikken-Seikagaku-Koza Vol. 1-Ⅰ, pp. 262-312, Tokyo Kagaku Dojin, Tokyo (in Japanese).

(8) K. Miyazaki and T. Saito (1981) : Isoelectrofocus by gel electrophoresis. "Basic methods for protein and enzyme" (ed. by T. Horio and N. Yamashita), pp. 333-347, Nankodo, Tokyo and Kyoto (in Japanese).

(9) N. Kuramitsu, K. Hamaguchi, and S. Tsunazawa (1979) : Protein structure. "Data Book in Biochemistry" (ed. by The Japanese Biochemical Society), pp. 174-175, Tokyo Kagaku Dojin, Tokyo (in Japanese).