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Purification of Eel Liver Glutamate Dehydrogenase Suffered Limited Proteolysis

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Abstract

Glutamate dehydrogenase (GDH) suffered limited proteolysis was purified from the acetone powder of the eel liver. Limited proteolysis of GDH occurred at heat treatment in purification procedures, but it was inhibited by leupeptin, an inhibitor of trypsin or thiol proteases. Furthermore, GDH suffered limited proteolysis during preservation of the liver at −20℃, but GDH purified from preserved livers, which was perfused with phosphate buffered saline containing leupeptin preliminarily, was almost native form. Molecular weight and pI of limited proteolyzed GDH were 340,000 and pH 5.9, respectively. There was no difference in molecular weight and isoelectric point between native and limited proteolyzed GDHs in oligomer state. However, subunit of GDH suffered limited proteolysis consisted of two kinds of proteins with different molecular weight. One was 53,000 to 55,000 dalton and the other 50,000 to 52,000 dalton. Native GDH consisted of only the former proteins with 53,000 to 55,000 dalton.

p-Chloromercuribenzoic acid inhibited the activity of GDH suffered limited proteolysis, but stimulated that of native GDH. The effect of allosteric effectors, ADP and GTP, on native GDH was more drastic than that on GDH suffered limited proteolysis.

Hypothesis for physiological meaning of GDH suffered limited proteolysis was also discussed.

When the livers preserved at −20 ℃ for several weeks were used for purification, glutamate dehydrogenase (GDH) suffered limited proteolysis was obtained. However, GDH was native and didn't suffer limited proteolysis if leupeptin, an inhibitor of proteases, was present during purification. There had been no reports so far about the occurrence *in vivo* of the limited proteolysis of GDH. Then, it is expected that the eel liver GDH is available for the investigation of the physiological meaning of the limited proteolysis. The limited proteolysis is thought as a process in the life-cycle of GDH, that is, from the synthesis of GDH to degradation to amino acids.

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There were reports on limited proteolysis of bovine liver GDH by chymotrypsin[1,2], *E. coli* pyruvate oxidase by chymotrypsin[3], and *E. coli* aspartase by trypsin[4]. All these reports were intended to reveal the structure and function of their enzymes by artificial limited proteolysis.

This paper describes the method of purification of the GDH suffered limited proteolysis, the comparison in enzymatic properties between the native GDH and limited proteolyzed GDH, and further discusses on the physiological meaning of limited proteolysis of GDH.

Materials and Methods

Materials

Fresh livers of eel were obtained from Sueyoshi Co. in Kagoshima. Protamine sulfate from salmon roe was purchased from Wako Pure Chemical Industries Ltd. ADP and GTP were purchased from Oriental Yeast Co. and Yamasa Soybean Co., respectively. GTP-Sepharose was prepared as described previously[5]. Other reagents were purchased from Nakarai Chemicals Ltd. and Wako Pure Chemical Industries.

Assay of GDH Activity

GDH activity was assayed as described previously[6]. Protein was assayed by the method of Bradford[7].

Procedures for the Purification of GDH Suffered Limited Proteolysis

Livers were perfused with phosphate buffered saline to remove blood and stocked at −20℃ for several weeks before use. All steps for purification was carried out at 7℃ exception extraction and heat-treatment procedures.

(*Preparation of Acetone Powder*) Acetone powder of eel livers stocked at −20℃ was prepared as described previously[5].

(*Extraction of GDH from Acetone Powder*)

One litre of 0.01 M Tris-Cl (pH 7.5)−1 mM EDTA was added to 16 g of acetone powder and stirred at room temperature for 1 h by a magnetic stirrer. Then the solution was centrifuged at 5,000 rpm for 30 min, and resulting supernatant was fractionated by addition of solid $(NH_4)_2SO_4$. Fraction with 25 to 60% saturated ammonium sulfate was collected and dialyzed against 0.01 M Tris-Cl (pH 7.5)−1 mM EDTA.

(*Heat-Treatment of* 25 *to* 60% $(NH_4)_2SO_4$ *Fraction*)

Protamine sulfate was added to the 25 to 60% $(NH_4)_2SO_4$ fraction, which was dialyzed as described above. Eleven mg of protamine sulfate was added to 100mg protein in the 25 to 60% $(NH_4)_2SO_4$ fraction. Then the ammonium sulfate fraction was incubated at 55℃ for about 10 min, cooled in ice water to room temperature and centrifuged at 35,000 rpm for 30 min. The supernatant was fractionated by addition of solid $(NH_4)_2SO_4$ and fraction with 30 to 50% $(NH_4)_2SO_4$ was collected.

(*Affinity Chromatography on GTP-Sepharose*)

Fraction with 30 to 50% $(NH_4)_2SO_4$ was dialyzed against 0.05 M Tris-Cl (pH 7.5)−1 mM EDTA adequately and the dialyzed solution was applied to GTP-Sepharose column (2 ×9.5cm) equilibrated with the same buffer used for dialysis. After washing the column with

0.05 M Tris-Cl (pH 7.5)−1 mM EDTA GDH was eluted by a linear gradient of KCl in the same buffer. GDH was eluted as a single peak and the obtained GDH was one suffered limited proteolysis.

Determination of Molecular Weight and Isoelectric Point

Molecular weight of the purified GDH was determined using a TSK 4000 SW column as described previously[6]. Molecular weight of the subunits of GDH was determined by the method of Laemmli[8]. Isoelectric point of GDH was determined as described previously[5].

Results and Discussion

Purification of GDH Suffered Limited Proteolysis

Table 1 shows the typical example of the purification of GDH suffered limited proteolysis. Specific activity of the GDH was always higher than that of the native GDH. After the heat treatment total activity of the GDH increased by 2.4 times higher than that of prior step. This increase was assumed due to the progress of the limited proteolysis of GDH. Even if acetone powder prepared from fresh livers was used for the purification of GDH, the limited proteolysis occurred at the heat treatment procedure. When this heat treatment was performed in the presence of leupeptin, purified GDH was native (Table 2).

GDH suffered limited proteolysis more intensely was observed when GDH was purified from the acetone powder prepared from livers preserved at −20℃ for several weeks. However, the GDH which was purified from the acetone powder prepared livers perfused with phosphate buffered saline containing leupeptin was almost native. This proved that the limited proteolysis of the native GDH progressed slowly during preservation at −20℃, and the limited proteolysis occurred not only during heat treatment, but also during preservation.

Some Properties of GDH Suffered Limited Proteolysis

The molecular weight and isoelectric point of the GDH suffered limited proteolysis was 340,000 and pH 5.9, respectively. There was no difference in molecular weight and isoelectric point between native and limited proteolyzed GDH in oligomer state as shown in

Table 1. Purification of the GDH suffered limited proteolysis.

Step	Vol	Protein		Activily		Recoverry
	(ml)	(mg/ml)	(mg)	(U/mg)	(U)	(%)
25−60%$(NH_4)_2SO_4$ Fraction	365	28.5	10402	0.039	406	100
Protamine and Heat−Treatment	342	19.2	6566	0.147	965	238
30−50%$(NH_4)_2SO_4$ Fraction	39	33.1	1291	1.112	1436	354
GTP−Sepharose	14.6	3.4	50	21.9	1086	268

Livers were perfused with Ringer solution and preserved at −20℃ for 1 month.
Livers ; 326.5g.

Table 3. However, limited proteolyzed GDH consisted of two kinds of subunits as shown in Fig. 1. One with molecular weight of 53,000 to 55,000 was the same as the subunit of native GDH, but the other with molecular weight of 50,000 to 52,000 was absent in native GDH. On the other hand, there were some significant differences of enzymatic properties. Native GDH had always a lower specific activity than that of limited proteolyzed GDH. The degree of the high specific activity of limited proteolyzed GDH was due to the degree of proteolysis. As shown in Table 4, the effect of allosteric effectors, ADP and GTP, on native GDH was more drastic than that on limited proteolyzed GDH. Furthermore, the activity of native GDH was stimulated and that of limited proteolyzed GDH was inhibited by *p*-chloromercuribenzoic acid (PCMB), which reacts with free SH-group of cystein residue in protein. These reverse results, stimuratory and inhibitory effects, were interesting for the different role of free SH-group in both GDHs. After PCMB treatment native GDH returned to the initial state in the presence of excess 2-mercaptoethanol, but GDH suffered limited proteolysis couldn't. It was assumed that the latter suffered irreversible structural change.

Table 2. Effect of starting material on the purification of glutamate dehydrogenase.

Starting Material	Limited Proteolysis
1. Mitochondrial Fraction	
−20℃ for 1−1.5 month	−
25℃ for 17 h	−
2. Acetone Powder of Eel Liver	
+Leupeptin at heat treatment	−
−Leupeptin at heat treatment	+
3. Acetone Powder of the Liver perfused with or without Leupeptin*	
+Leupeptin	−
−Leupeptin	+

*Livers were preserved at −20℃ for 1−1.5 month after removing blood.

Table 3. Comparison of molecular weight and pI between limited proteolyzed and native glutamate dehydrogenase.

M.W. & pI	Limited proteolyzed GDH	Native GDH
M.W.	340,000	340,000
pI	5.9	5.9
M.W. of Subunit	53,000−55,000 50,000−52,000	53,000−55,000

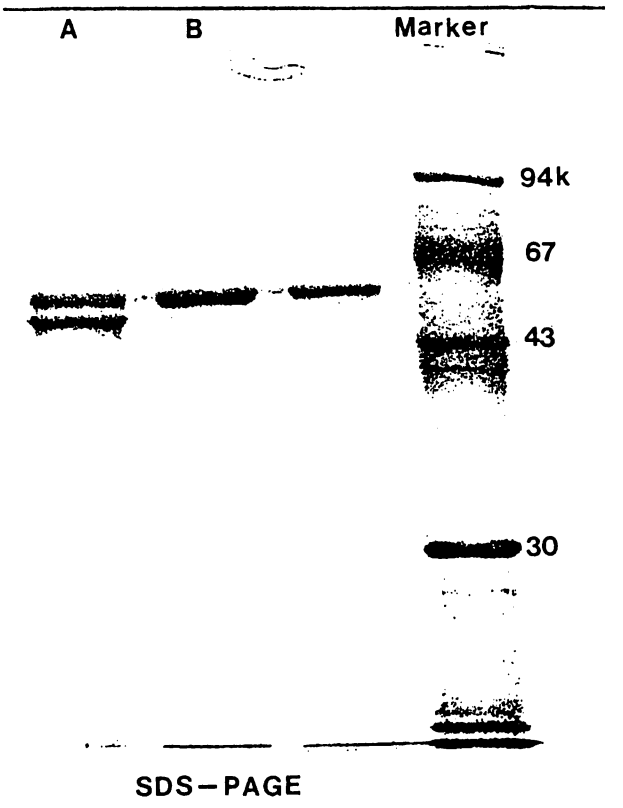

Fig. 1. Polyacrylamide gel electrophoresis of limited proteolyzed (A) and native GDH (B) in the presence of sodiumdodecyl sulfate and 2-mercaptoethanol.

Hypothesis for Physiological Meaning of GDH Suffered Limited Proteolysis

Fig. 2 shows the hypothetical life cycle of GDH. It has been revealed that GDH with signal peptide, which was biosynthesized in ribosome, recognizes the mitochondrial membrane[9,10]. The signal peptide is digested by protease on mitochondrial membrane, which is not yet isolated, then GDH enters into mitochondrial matrix. The native GDH enters into mitochondrial matrix. The native GDH described in this paper is GDH in mitochondrial matrix.

Although each of intracellular proteins has individual length of life and is degraded, it has been accepted generally that its final stage of the degradation to amino acids is performed in lysosome. Similarly GDH of eel liver is thought to be degraded to amino acids in the lysosome, but it is assumed that the native GDH suffers limited proteolysis during the process moving from matrix to lysosome. The limited proteolysis of the native GDH is thought to be an initial step for the degradation of GDH.

Table 4. Comparison of enzymatic properties between native GDH and GDH suffered limited proteolysis.

PCMB Treatment	Addition (mM)	GDH Native U/ml (%)		Limited Proteolysis U/ml (%)	
Before	None	7.93	(100)	7.58	(100)
	ADP (1)	69.3	(874)	19.2	(254)
	GTP (1)	0.23	(2.9)	0.54	(7.1)
After	None	23.8	(300)	4.49	(59)
	ADP (1)	38.3	(482)	6.29	(83)
	2-ME (19)	8.40	(106)	5.31	(70)
	2-ME+ADP	64.4	(812)	13.1	(172)
	2-ME+GTP	0.12	(2)	1.05	(14)

PCMB Treatment ; GDH in 0.5mM PCMB – 0.025M Tris-Cl (pH7.5) 30℃, 10 min
Native GDH ; 14.6 U/mg.
GDH suffering limited proteolysis ; 37.0 U/mg.
2-ME ; 2-mercaptoethanol.

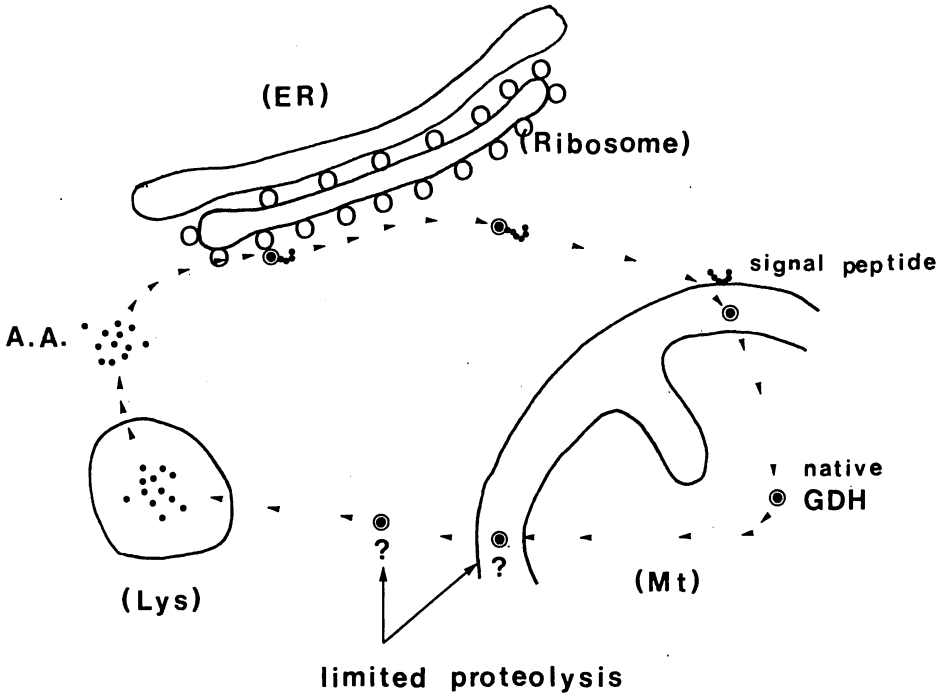

Fig. 2. Hypothetical life cycle of GDH. ER, endoplasmic reticulum ; Mt, mitochondria ; Lys, lysosome ; A.A., amino acids.

There was a report on group-specific proteinases for apoproteins of pyridoxal enzymes[11]. The report discussed the role of group-specific proteinases for the initial step of degradation of pyridoxal enzymes. If the protease which causes limited proteolysis of native GDH is purified from the eel liver and its enzymatic properties and cellular localization are revealed, the meaning of the GDH suffered limited proteolysis will be revealed. The purified protease itself is expected to become an available tool to investigate the structure and function of GDH.

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