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Thermalstability of the Glutamate Dehydrogenase of Eel Liver

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Abstract

Glutamate dehydrogenase purified from the eel liver was not inactivated at 55°C for 5min. in the presence of the crude extract of the eel liver, but inactivated in the absence of the crude extract. Protective material in the crude extract was not removed by dialysis and was most found in the supernatant after $(NH_4)_2SO_4$ precipitation (0–40 %) of the crude extract treated with heating process, in this supernatant the enzyme activity was absent.

After the heating process in the purification procedure, the specific activity of the enzyme increased 2–to 4–fold higher than that of the enzyme before the heat treatment and the recoveries of the activity were usually from 100 to 250 %. However the enzyme extracted from the eel liver obtained at September or October could not be purified by heating process.

Thermalstabilities of glutamate dehydrogenase (EC 1. 4. 1. 2–4, GDH) purified from mammals to microorganisms vary greatly. Bovine liver GDH, which is most extensively investigated, is stable for an hour at 41°C in 0. 2 M phosphate buffer (pH 7. 6), but loses activity rapidly and irreversibly at 50°C[1] unless sodium sulfate (0. 5 M) is added to the buffer[2]. It is known that $NADP^+$-specific GDH from *Neurospora* retains activity for several days at 50°C and pH 7. 2[3]. However NAD^+-specific GDH from *Neurospora* is inactivated rapidly at raised temperatures and low ionic strengths and is more stable in the presence of NAD^+ and the competitive inhibitor of isophthalate[4]. NAD^+-specific GDH's from lower fungi are also reported to be unstable[5], but those isolated from *Clostridium* SB_4[6] and *Peptococcus aerogenes*[7] are stable at 50°C, and so are the $NADP^+$-dependent enzymes of *Salmonella*[8] and *E. coli*[9].

Heat treatment at 55°C for about 10 min. was performed to purify GDH from the eel liver. This treatment increased the specific activity of the enzyme by 2- to 4-fold, comparing with that of the enzyme before the treatment. This report describes the thermalstability of GDH purified from the liver of the eel (*Anguilla japonica*) and the presence of some material in the crude extract which has protective effect on the heat denaturation of the enzyme.

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Experimental procedure

Enzyme assay The enzyme activity was measured at 30°C in 4 ml of 0.1 M Tris-Cl buffer (pH 8.0) containing 2.5×10^{-4}M EDTA, 0.1 M ammonium chloride, 5×10^{-3}M α-ketoglutarate and 1.5×10^{-4}M NADH. The oxidation of NADH was followed by the decrease in absorbance at 340 nm with a Hitachi EPU-2 spectrophotometer. One unit is defined as the amount of the enzyme catalyzing a decrease of one micromole of NADH per minute under the assay condition described above.

In the reverse reaction the assay mixture (4 ml) was 0.1 M glutamate and 5×10^{-3}M NAD^+ in 0.16 M Tris-Cl buffer (pH 8.5).

Protein concentration was determined by the method of Lowry *et al.*[10].

Enzyme source Cultured eels weighing about 150 g were used and the eel livers were pooled from 100 to 150 eels to prepar the acetone powder.

Purification procedure The acetone powder was prepared according to the method described previously[11]. Crude extract was obtained by extracting the acetone powder with 10 volumes of 0.01 M Tris-Cl buffer (pH 8.0) containing 10^{-3} M EDTA at room temperature for one hour. After the enzyme precipitated in a 25 to 60% $(NH_4)_2SO_4$ cut was dialyzed, the dialyzed solution was heated at 55°C for approximately 10 min. Precipitated protein was removed by centrifugation and the enzyme in the supernatant was precipitated in a 30 to 40% $(NH_4)_2SO_4$ cut. Then the enzyme was purified with GTP-Sepharose, of which preparation was described previously[11].

The homogeneity of the enzyme was recognized as described in another paper[12]. The purification procedure is summerized in Table 1.

Table 1. Summary of the purification of the eel liver glutamate dehydrogenase

Step	Volume (ml)	Protein (mg)	Specific Activity (unit/mg)	Activity (units)	Recovery (%)
25-60% $(NH_4)_2SO_4$	482.0	5145.3	0.35	1800.1	100
Protamine and Heat treatment	413.0	3799.6	0.93	3529.8	196
30-40% $(NH_4)_2SO_4$	36.5	383.6	5.57	2134.7	119
GTP-Sepharose	30.0	9.8	73.29	718.2	40

Wet weight of eel livers, 435.7 g

Materials Co-enzymes, NAD^+ and NADH were obtained from Oriental Yeast Co. CNBr-activated Sepharose was perchased from Pharmacia Fine Chemicals and other chemicals from Wako Pure Chemical Industries.

Results

Effect of substrates on the thermalstability of purified GDH The enzyme in 0.1 M Tris–Cl buffer (pH 8.0) containing 10^{-3}M EDTA was incubated in the presence or absence of substrates at 30, 35, 40, 50 and 60°C for 15 min. After the incubation the enzyme was immediately transfered to an icebath and the activity was assayed at 30°C. In the absence of substrates, the enzyme was inactivated at raised temperature (Fig. 1). In the presence of 0.1 M ammonium chloride and 5×10^{-3}M α-ketoglutarate, the enzyme was stable at 40°C but inactivated at 55°C almost completely. Glutamate (0.1 M) as one of substrates had not protective effect on the heat denaturation of the enzyme.

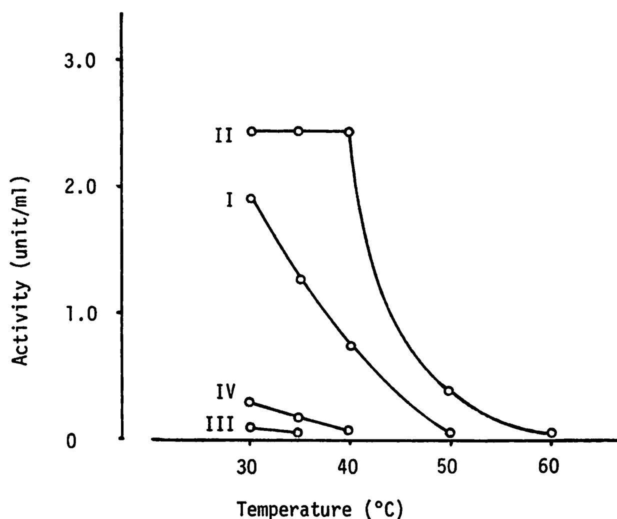

Fig. 1 Effect of substrates on the thermalstability of the purified glutamate dehydrogenase.

I, II: After the enzyme was incubated in the absence (I) or the presence (II) of α-ketoglutarate and NH_4Cl, its activlty was assayed in Tris-Cl buffer (pH 8.0) contanining EDTA, α-ketoglutarate, NH_4Cl and NADH.

III, IV: After the enzyme was incubated in the absence (III) or the presence (IV) of glutamate, its activity was assayed in Tris-Cl buffer (ph 8.5) containing glutamate and NAD^+.

Effect of crude extract on thermalstability of the purified GDH As shown in Fig. 1., the purified enzyme was inactivated at 55°C for 15 min. However the enzyme was stable in the crude extract at 55°C. The recovery of the enzyme activity after the heat treatment was approximately 200 % (Table 1). Then the effect of the crude extract on the thermalstability of the purified GDH was investigated. After the heating process the crude extract was centrifuged, and the supernatant was

then fractionated with ammonium sulfate, 0–30 % (Fraction I), 30–40 % (Fraction II) $(NH_4)_2SO_4$ fraction and the leaving fraction (Fraction III) unprecipitated with 40 % saturated ammonium sulfate. Each fraction was adequately dialyzed against 0.1 M Tris-Cl buffer (pH 7.5) containing 10^{-3}M EDTA.

The protein concentration and activity of the enzyme in each fraction were shown in Table 2-A. The purified GDH (50 μl) was added to each fraction, of which concentration of protein was adjusted to equal amount of protein (250 μg), and incubated at 55°C for 5 min. After the incubation the incubation mixture was immediately transfered to an icebath and the enzyme activity was assayed at 30°C.

Table 2. Effect of crude extract on the thermalstability of the purified glutamate dehydrogenase

A. Activities of GDH in Fraction I, II and III

	Purified	Fraction		
	GDH	I	II	III
Specific activity (unit/mg)	54	0.29	2.20	0
Protein (mg/ml)	0.015	5.03	12.9	2.50

B. Effect of Fraction I, II and III on the thermalstability of GDH

	Activity (ΔO.D./min)			
	Control	A	B	C
Before heat treatment	0.073	0.056	0.144	0.067
After heat treatment	0	0	0.027	0.044
After / Before (%)	0	0	19	65

Control: 50 μl purified GDH + 150 μl 0.1 M Tris-Cl (pH 7.5)
A: 〃 + 110 μl 〃 + 40 μl Fraction I
B: 〃 + 130 μl 〃 + 20 μl Fraction II
C: 〃 + 50 μl 〃 + 100 μl Fraction III

As shown in Table 2-B Fraction III had most protective effect on the heat denaturation of the enzyme, the enzyme activity was retained 65 % of the initial activity. However the purified enzyme was inactivated completely at 55°C for 5 min. Fraction I had no effect and Fraction II had a little effect on protection.

Seasonal changes of the thermalstability of the crude enzyme Crude enzyme was extracted from the acetone powder which was prepared from 100 to 150 eel livers. Eels used were almost same size and same weight (about 150 g) at any season. However crude enzyme prepared at September and October was inactivated by heat treatment. Then seasonal changes of the effect of heat treatment on the purification were investigated.

Specific activity of the crude enzyme, 25 to 60 % $(NH_4)_2SO_4$ fraction, changed

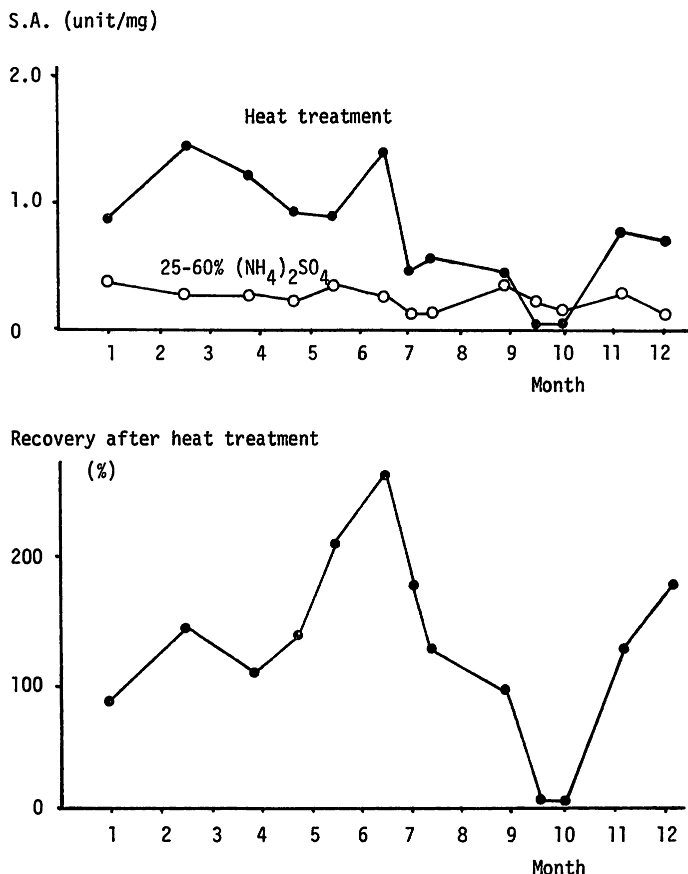

Fig. 2 Seasonal changes of the thermostability of the crude enzme.

slightly throughout the year (Fig. 2). After the heat treatment the specific activities of the enzyme prepared at any month except September and October were raised 2 to 4 times higher than those of crude enzyme before the heat treatment. The recoveries of these enzyme activities after the heat treatment were 100 to 250 % but those at September and October were below 10 %.

Discussion

Purified GDH in 0.1 M Tris-Cl (pH 7.5) containing 10^{-3}M EDTA was inactivated comletely by heat treatment, at 55°C for 5 min. In the presence of α-ketoglutarate and ammonium chloride the enzyme increased its thermalstability and tolerated at 40°C for 15 min. However in the presence of glutamate the enzyme did not increase its thermalstability. It is assumed that relative high concentration of ammonium chloride (0.1 M) and α-ketoglutarate made the enzyme increase its thermalstability. In bovine liver GDH high concentration of sodium

sulfate (0. 5 M) makes the enzyme increase its thermalstability[2]. Phosphate buffer (pH 7. 6, 0. 2 M) also had same effect on thermalstability of bovine liver GDH[1].

As shown in Table 2, it was found that in the crude extract of the eel liver there was some protective compound to make the eel liver GDH increase its thermalstability. This compound seems to be a material having high molecular weight like protein, since it was not removed by dialysis. Because of the presence of this protective compound, the heat treatment during the purification procedure was very useful to remove other proteins except GDH as shown in Table 1.

$NADP^+$-specific GDH's of *Salmonella*[8], *Neurospora*[3] and *E. coli*[9] are purified by heating process, at 55°C for 5 min., 53°C for 30 min. and 60°C for 10 min., respectively. NAD^+-specific GDH of *Clostridium* SB_4[2] is also purified by heat tratment at 65°C for 15 min. However these enzymes purified from microorganisms described above are stable at 55 to 60°C without addition of some materials to protect against the denaturation of the enzyme.

It is reported by Rogers[13] that the loss of the activity of bovine liver GDH during the heating process did not occur from successive partial denaturation of all the enzyme molecules which were present and soluble at the beginning of the heating process, and that the heat treatment had not altered the affinity of the substrate binding site for isophthalic acid, as competitive inhibitor, in the remaining "active" enzyme molecules. If these results reported by Rogers are true for other thermostable GDH's, these enzymes maintain to have their original properties after the heating process.

Seasonal changes of the thermalstability of the crude enzyme were obserbed. Crude enzyme prepared at September and October was inactivated by heat treatment. After the heat treatment the specific activities of the enzyme prepared at any month except September and October were raised 2 to 4 times higher than those of crude enzyme before the heat treatment. It is assumed that the protective compound to make the eel liver GDH increase its thermalstability was absent in the extract of the eel liver obtained at September and October. The absence of the protective compound seems due to the change of the components in the eel liver by the changes of feeding or the preparation for migration, eels in Kagoshima migrate from river to the sea at October or November. Lewander *et al.*[14] reported the difference of liver protein content between the yellow and silver phases of the European eel, the yellow eel is successively changed towards the silver eel phase during the summer and autumn of the year of spawning migration.

References

1) Olson, J. A. and Anfinsen, C. R. (1953): Kinetic and equilibrium studies on crystalline L-glutamic acid dehydrogenase. *J. Biol. Chem.*, **202**, 841-856.

2) Kubo, H., Iwatsubo, M., Watari, H. and Soyama T. (1959): Sur la polymerization

et la forme moleculaire de la glutamico-deshydrogenase. *J. Biochem.*, **46**, 1171–1185.

3) BARRETT, R. W. and STRICKLAND, W. N. (1963): Purification and characterization of a TPN-specific glutamic acid dehydrogenase from *Neurospora crassa*. *Arch. Biochem. Biophys.*, **102**, 66–76.
4) VERONESE, F. M., NYC, J. F., DEGANI, Y., BROWN, D. M. and SMITH, E. L. (1974): Nicotinamide adenine dinucleotide-specific glutamate dehydrogenase of *Neurospora* I. *J. Biol. Chem.*, **249**, 7922–7928.
5) LEJOHN, H. B. and STERENSON, R. M. (1970): Multiple regulatory processes in nicotinamide adenine dinuleotide-specific glutamate dehydrogenases. *J. Biol. Chem.*, **245**, 3890–3900.
6) WINNACKER, E. L. and BARKER, H. A. (1970): Purification and properties of a NAD-dependent glutamate dehydrogenase from *Closridium* SB_4. *Biochim. Biophys. Acta*. **212**, 225–242.
7) JOHANSON, W. M. and WESTLAKE, D. W. S. (1972): Purification and characterization of glutamic acid dehydrogenase and α-ketoglutaric acid reductase from *Peptococcus aerogenes*. *Can. J. Microbiol.*, **18**, 881–892.
8) COULTON, J. W. and KAPOOR, M. (1973): Purification and some properties of the glutamate dehydrogenase of *Salmonella typhimurium*. *Can. J. Microbiol.*, **19**: 427–438.
9) VERONESE, F. M., BOCCU, E. and CONVENTI, L. (1975): Glutamate dehydrogenase from *Escherichia coli*: Induction, purification and properties of the enzyme. *Biochim. Biophys. Acta*. **377**, 217–228.
10) LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RAFALL, R. J. (1951): Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
11) HAYASHI, S. and OOSHIRO, Z. (1977): Availability of GTP-Sepharose for the purification of glutamate dehydrogenase from eel liver. *Bull. Japan. Soc. Sci. Fish.*, **43**, 107–113.
12) HAYASHI, S. and OOSHIRO, Z. in preparation
13) ROGERS, K. S. (1975): Heat denaturation of bovine liver glutamate dehydrogenase. *Proc. Soc. Exper. Biol. Med.*, **149**, 1023–1025.
14) LEWANDER, K., DAVE, G., JOHANSSON, M.-L., LARSSON, A. and LIDMAN, U. (1974): Metabolic and hematological studies on the yellow and silver phases of the European eel, *Anguilla anguilla* L. I. *Comp. Biochem. Physiol.*, **47 B**, 571–581.