PRELIMINARY STUDIES IN SOME ASPECTS OF AMINO ACID BIOSYNTHESIS IN JUVENILES OF *PENAEUS MONODON* FABRICIUS[+]

II. PARTIAL PURIFICATION AND CHARACTERIZATION OF MUSCLE L-GLUTAMATE DEHYDROGENASE.

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ABSTRACT

A method of partially purifying L-glutamate dehydrogenase, a key enzyme in the biosynthesis of non-essential amino acids, from the muscle of *P. monodon* juveniles is presented. Enzyme extraction with Tris-acetate buffer, ammonium sulfate fractionation, DEAE-cellulose chromatography, and Sepharose 4B chromatography were used. A 260-fold purification was obtained, but the recovery of enzyme activity was low. Possible modification in the method and the need for further purification are discussed.

P. monodon possesses an enzyme which catalyzes the *de novo* synthesis of glutamate from alpha-ketoglutarate and ammonia. The enzyme has a pH optimum for the reductive amination of alpha-ketoglutarate between pH 8.0 and 8.2. The low Michaelis-Menten constant (1.03×10^{-4}M) of the enzyme for alpha-ketoglutarate strongly suggests the physiological importance of this pathway in *P. monodon*. Substrate inhibition by alpha-ketoglutarate at high non-physiological levels was observed and discussed.

The molecular weight of the native enzyme estimated by molecular sieve chromatography is 320,000 daltons. Subunit studies using SDS polyacrylamide gel electrophoresis suggest that the enzyme possesses a unique molecular organization compared to those derived from other sources.

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INTRODUCTION

The occurrence and biosynthesis of all non-essential amino acids from other precursors will require a pathway for the formation of alpha-amino groups . The main pathway for *de novo* generation of alpha-amino groups from ammonia is through the reductive amination of alpha-ketoglutarate catalyzed by L-glutamate dehydrogenase [L-glutamate: NAD (P) oxidoreductase E. C. (1.4.1.3)] as in the following reaction:

$$\text{alpha-ketoglutarate} + NH_4^+ + \text{NAD (P) H} \rightleftharpoons \text{glutamate} + \text{NAD (P)}^+ + H_2O$$

The amino group incorporated into glutamate is then transferred to various alpha-keto acids in specific transamination reactions giving rise to the corresponding amino acids.

Thermodynamic data obtained by Subramanian (1) suggest that the forward reaction is more favored than the reverse reaction as written above. Reductive amination is accompanied by large negative free energy change ($\triangle G^o$) which results from both a large negative enthalpy change ($\triangle H^o$) and a moderate positive entropy change ($\triangle S^o$).

Glutamate dehydrogenase (GDH) is a key enzyme that provides an important link between energy and nitrogen metabolism. It regulates the entrance of carbon into the tricarboxylic acid cycle by controlling the level of alpha-ketoglutarate. This is best regulated by the energy status of the cell since the reaction requires chemical energy in the form of the reduced co-enzyme, NADH. Furthermore, it plays a significant role in nitrogen metabolism by controlling the entrance of ammonia into the metabolism and facilitating its release from glutamate (2).

The mammalian enzyme has been exhaustively studied (See review by Eisenberg, Josephs and Reisler, 1976) (3). Glutamate dehydrogenase has also been isolated from frog and tadpole (4), dogfish and chicken liver (5) and yeast (6).

Subtle and quite marked differences exist among enzyme from a large number of sources. Active bovine GDH has a molecular weight of about 270,000 daltons from ultracentrifuge and molecular sieve data (7). Light scattering data reveal that four monomer units comprise the polymer (8). On the other hand, the frog liver enzyme has a molecular weight of about 250,000 daltons. It is a dimer which migrates as one band towards the anode on polyacrylamide gels at pH 8.4. Light scattering and ultracentrifuge data reveal that the dogfish liver enzyme has a molecular weight of about 330,000 daltons. No data pertaining to its subunit nature was found.

The Km values for the different substrates involved in the reductive amination of alpha-ketoglutarate and the oxidative deamination of glutamate

by the enzyme from various sources have been obtained (4, 5, 7, 8). The affinity of the substrates of the reductive amination reaction for the bovine liver enzyme appears to be greater than that of the substrates of the reverse reaction for the enzyme. In contrast, the dogfish and frog liver enzymes exhibit behaviour contrary to that of the bovine enzyme.

Most of our present knowledge on the structure and function of GDH arise from the extensive studies done on the bovine liver enzyme. The presence of glutamate dehydrogenase has also been demonstrated in Crustacea (9). This paper describes the occurrence, partial purification and characterization of glutamate dehydrogenase from the muscle of the prawn, *Penaeus monodon.* The enzyme was isolated and partially purified by extraction with Tris-acetate buffer, ammonium sulfate fractionation, DEAE-cellulose chromatography and gel filtration on Sepharose 4B. Its pH optimum for the reductive amination reaction, apparent Michaelis constant for alpha-ketoglutarate, native molecular weight and subunit nature were studied.

L-glutamate dehydrogenase activity is an important control site because of its role in the redistribution of alpha-amino groups from amino acids present in excess to those present in limited quantities. Its demonstration and characterization in *P. monodon* is significant not only in comparative biochemistry, but also to the elucidation of the precise metabolic role of the enzyme in the intact animal.

MATERIALS AND METHODS

Animals – Juveniles of the prawn, *Penaeus monodon,* the source of all glutamate dehydrogenase preparations, were obtained from the Leganes Research Station of the Southeast Asian Fisheries Development Center, Iloilo, Philippines. The tail-end muscle of freshly killed animals were dissected out and immediately frozen. Enzyme extraction was done on the frozen thawed samples.

GDH Activity Assay – Prawn muscle extracts and eluates from column chromatography were assayed at 27°C for glutamate dehydrogenase activity as previously described by Fahien and Cohen, 1970 (4). One unit of activity is defined as the amount of enzyme that catalyzes the oxidation of 1 micromole of NADH per min. under specified conditions. Specific activity is defined as units activity per mg of protein. Total protein was determined by using the method of Lowry, *et al.,* (10).

Partial Purification of GDH – The method used involved the extraction of the enzyme from prawn muscle tissue by homogenization in Tris-acetate buffer, sonication and subsequent centrifugation at 38,000 x g for 1 hour. It was followed by ammonium sulfate fractionation, DEAE-cellulose chromatography and, gel filtration on Sepharose 4B.

The purification method used differed from that of Corman and

Inamdar for dogfish and chicken liver GDH (5) on the following features: (1) replacement of acetone precipitation by extraction with Tris-acetate buffer and subsequent centrifugation of 38,000 x g for 1 hour; (2) deletion of sodium sulfate precipitation and heat denaturation steps; and (3) substitution of Sepharose 4B chromatography for Sephadex G-100 chromatography.

All centrifugations were done using the SS-34 rotor of RC2-B refrigerated Sorvall centrifuge. Ion-exchange and molecular sieve chromatography were done at 0 to 4°C, unless specified otherwise.

Muscle tissue from prawn was suspended in 0.1M Tris-acetate – 0.01 mM EDTA, pH8.1. The suspension was homogenized in a Potter-Elvehjem homogenizer 12 strokes, sonicated for 2 min using 15 sec bursts and centrifuged at 38,000 x g for 1 hour. The supernate was collected and designated as Fraction I.

Solid ammonium sulfate was used to fractionate Fraction I to obtain 10%, 40%, 60% and 100% saturation. The precipitates obtained were dissolved in a minimum amount of 0.02 M Tris-chloride – 0.1mM EDTA buffer, pH8. To achieve maximal enzyme recovery, the 10 to 40% and the 40 to 60% fractions were separately dialyzed overnight against 0.02M Tris-chloride – 0.1mM EDTA buffer pH8 to remove excess ammonium sulfate and then pooled. The pooled fractions were designated as Fraction II.

Fraction II was then applied on an equilibrated DEAE-Cellulose (Bio-Rad Laboratories, Richmond, California) column (2.2 x 58 cm) with a bed height of 16.7 cm. The column was eluted with a sodium chloride gradient of 0 to 1M in a 0.02 M Tris-chloride – 0.1 mM EDTA buffer pH8.1. Fractions were collected and assayed for GDH activity and protein. Fractions with specific activities greater than 0.2 U/mg protein were combined.

Polycrylamide disc gel electrophoresis of the active fractions still showed impurities. Fraction III was concentrated with polyethylene glycol MW 20,000 (Sigma Chemical Co., St. Louis, Missouri) and redissolved in 0.02 M Tris chloride-0.1 mM EDTA-0.01M NaC1 buffer, pH 8.1 with sonication. Two milliliters of this solution containing 5.6 mg protein was applied to an equilibrated Sepharose 4B (supplied pre-swollen by Pharmacia Fine Chemicals, Uppsala, Sweden) column (1.3 x 50 cm) with a bed height of 40 cm and eluted with Tris-chloride-EDTA-NaCl buffer.

Disc Gel Electrophoresis – The polyacrylamide gel used was prepared according to the formulations furnished by Canalco Industrial Corporation for Research Disc Standard (RDS) gels. Electrophoresis was done at 4 ma per tube. After electrophoresis, the gels were stained using Coomasie Brilliant Blue R (Sigma Chemical Co.).

Sodium Dodecyl Sulfate (SDS) Gel Electrophoresis – Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Sigma Chemical Co.) was done following the procedure of Weber and Osborn, 1969 (12).

RESULTS

Rationale for choice of the muscle tissue

It was originally proposed to study the nature of the glutamate dehydrogenase enzyme found in the hepatopancreas of the juvenile prawns. However, enzyme assays showed no significant GDH activity in the hepatopancreas. Muscle tissue, on one hand, demonstrated higher levels of enzyme activity. Total proteolytic activity is 60-fold greater in the hepatopancreas than in the muscle suggesting that prawn hepatopancreas may be more actively involved in digestion rather than metabolism. Growth in this animal largely depends on metabolic transformations occuring in other tissues like the blood and the muscles. In an attempt to isolate more protein for purification and characterization, attention was shifted to the tail-end muscle tissue which showed higher GDH activity.

Enzyme extraction from prawn muscle and ammonium sulfate fractionation

The enzyme crude extract gave specific activities that ranged from 0.007 to 0.010 U/mg (Fraction I).

Preliminary fractionation experiments showed highest specific enzyme activity in the 10 to 40% ammonium sulfate fraction (specific activity of 0.030 U/mg). It appears that the enzyme is precipitated at about 35 to 45% ammonium sulfate saturation. Therefore, to achieve maximal enzyme recovery, the 10 to 40% and the 40 to 60% fractions were pooled. The fractions were separately dialyzed overnight against 0.02 M Tris-chloride, pH 8.1-0.1mM EDTA buffer to remove excess ammonium sulfate, then pooled (Fraction II). There was a 50% decrease in activity after dialysis.

DEAE-cellulose chromatography

The elution profile of Fraction II on DEAE-cellulose after passing Fraction II on an equilibrated column is shown in Figure 1. Fractions with specific activities greater than 0.2 U/mg were combined as Fraction III and used for gel filtration. A 33-fold purification was approximately achieved.

Sepharose 4B chromatography

Since the active fractions from DEAE-cellulose chromatography still showed some impurities as seen in Figure 6, a further purification step was required.

The elution profile on Sepharose 4B in Figure 2 demonstrates that the amount of protein eluted in the active fraction (Fraction IV) was one-eight of the total. Purification at this point was about 260-fold.

The data revealed that Sepharose 4B can be used to further purify the enzyme and gave a good estimate of its native molecular weight. There was an 8-fold decrease in the protein concentration of the active fractions.

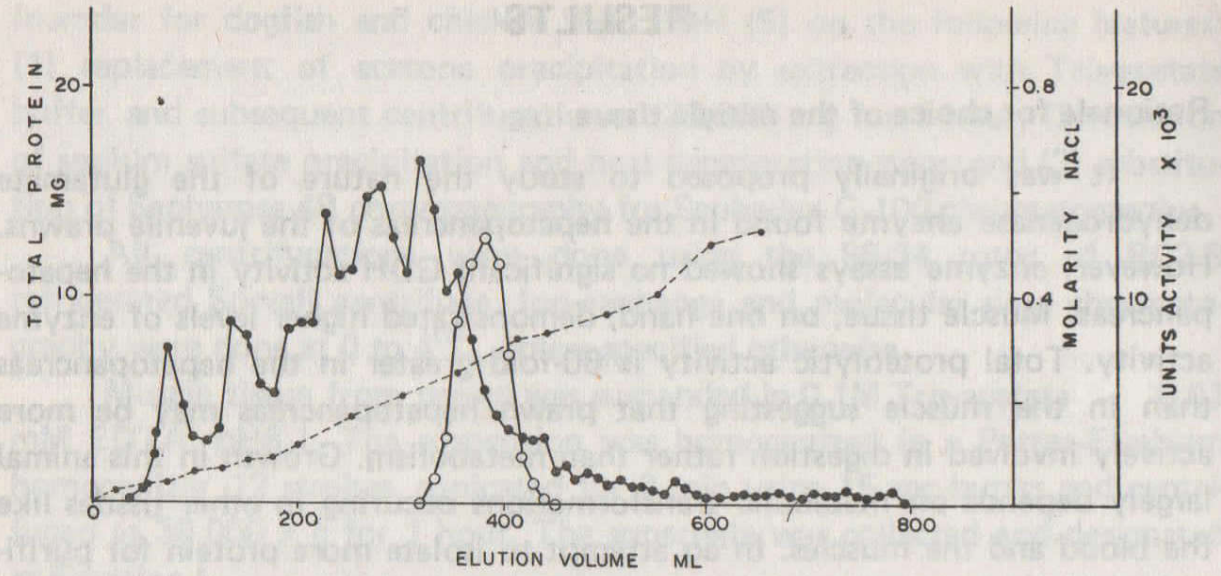

Figure 1. Protein and activity profile of Fraction II after DEAE-Cellulose chromatography.

total protein ●—●—●

units activity of G D H ○—○—○

sodium chloride concentration ●- - -●- - -●

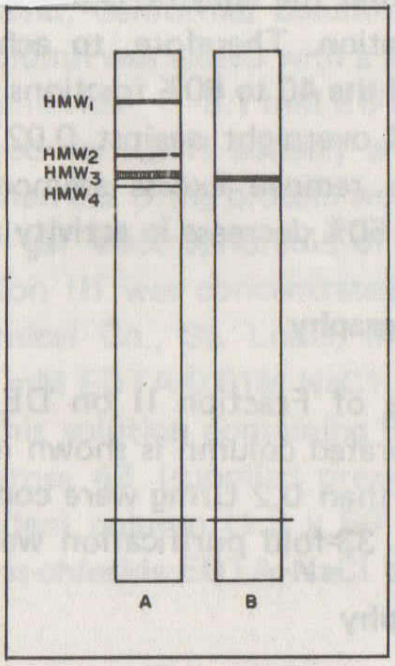

Figure 6. Polyacrylamide disc gel electrophoretic patterns of Sepharose 4B pool stained with NBT/PMS and Coomasie Brilliant Blue as described in Materials and Methods.
A — Stained with Coomasie Brilliant Blue
B — Stained with NBT/PMS

A summary of the purification obtained from Fraction I to Fraction IV is shown in Table I. However, the recovery of enzymic activity appears to be low.

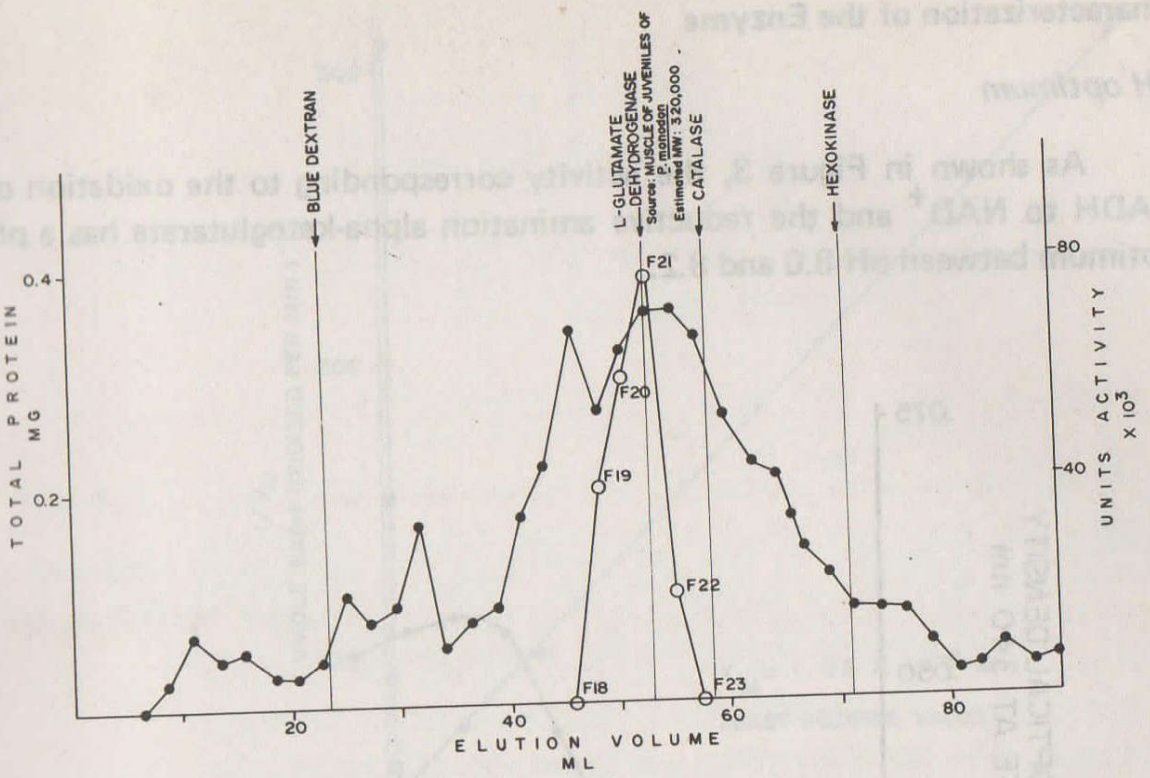

Figure 2. Protein and activity profile of Fraction III after Sepharose 4B chromatography.

●——● total protein
○——○ units activity of GDH
Refer to Materials and Methods.

The molecular weight of the native GDH from prawn muscle was computed from a plot of Ve/Vo against log MW, using 2 mg of catalase, hexokinase, and blue dextran as standards. The native molecular weight of the enzyme was determined to be about 320,000 daltons.

Table 1. Partial purification of glutamate dehydrogenase from prawn muscle. Refer to Materials & Methods.

Fraction	Total Protein mg	Total Activity Units	Average Specific Activity Units/mg x 10^3	Yield %	Purification Peak Fraction	Purification Ave.
I. Extract	1800	17.2	9.55	100		
II. Ammonium sulfate	500	12.7	25.40	74	4	3
III. DEAE-Cellulose	28	7.5	267.90	44	33	30
IV. Sepharose 4_B	2	0.3	150.00*	2	260**	220

* Loss of enzyme activity due to standing prior to this step resulted in lower specific activity in the last fraction compared to Fraction III.

** Computed on the basis of the relative amount of protein recovered in the peak fraction.

Characterization of the Enzyme

pH optimum

As shown in Figure 3, the activity corresponding to the oxidation of NADH to NAD^+ and the reductive amination alpha-ketoglutarate has a pH optimum between pH 8.0 and 8.2.

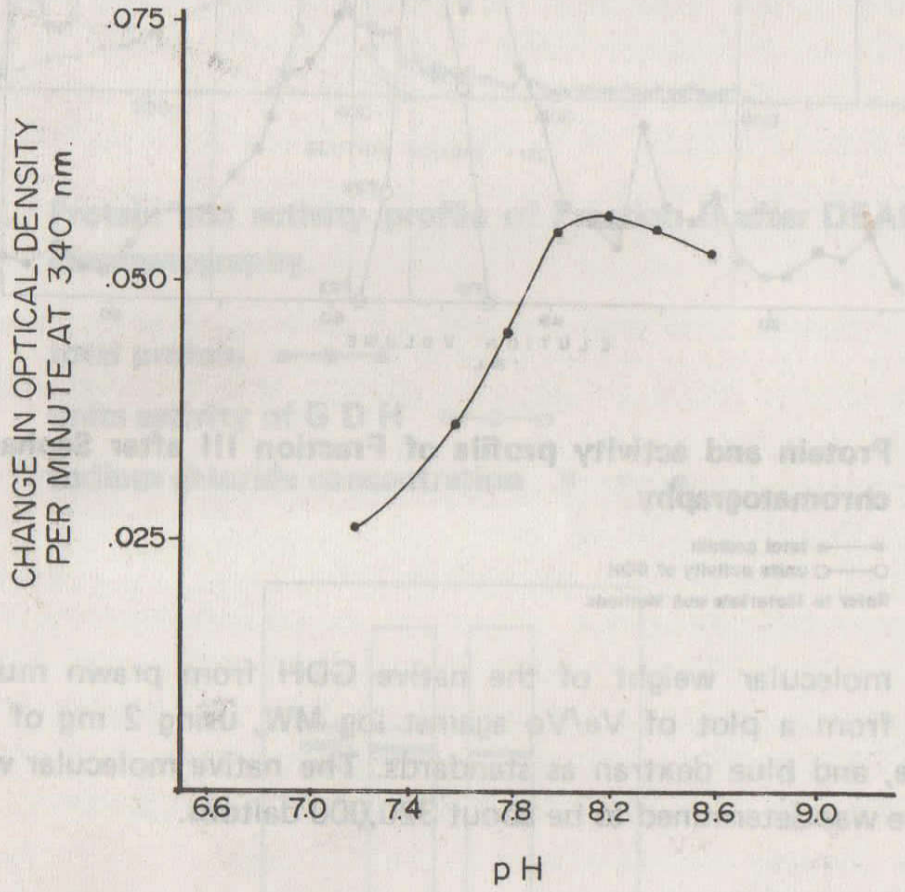

Figure 3. pH activity profile of crude glutamate dehydrogenase in Tris-acetate-EDTA buffer. Refer to Materials and Methods for the assay conditions.

Apparent K_m value

The Lineweaver-Burk plot (Figure 4) is linear up to 1 mM concentration of alpha-ketoglutarate beyond which significant substrate inhibition at high, non-physiological (greater than 10 times the K_m value) concentrations of substrate is noticeable.

K_m value obtained by the extrapolation of the Lineweaver – Burk plot was 1.03×10^{-4}M. This apparent constant compares well with the bovine liver enzyme at pH 7.6 in 0.5 M potassium phosphate buffer, 1.23×10^{-4} M (13), the dogfish liver enzyme at pH 8.0 in 0.01 M Tris-acetate buffer – 0.1 mM EDTA, 5.0×10^{-4}M (5), and the frog liver enzyme at pH 8.0 in 0.01 M Tris-acetate buffer – 0.1 mM EDTA, 5.0×10^{-3} M (4).

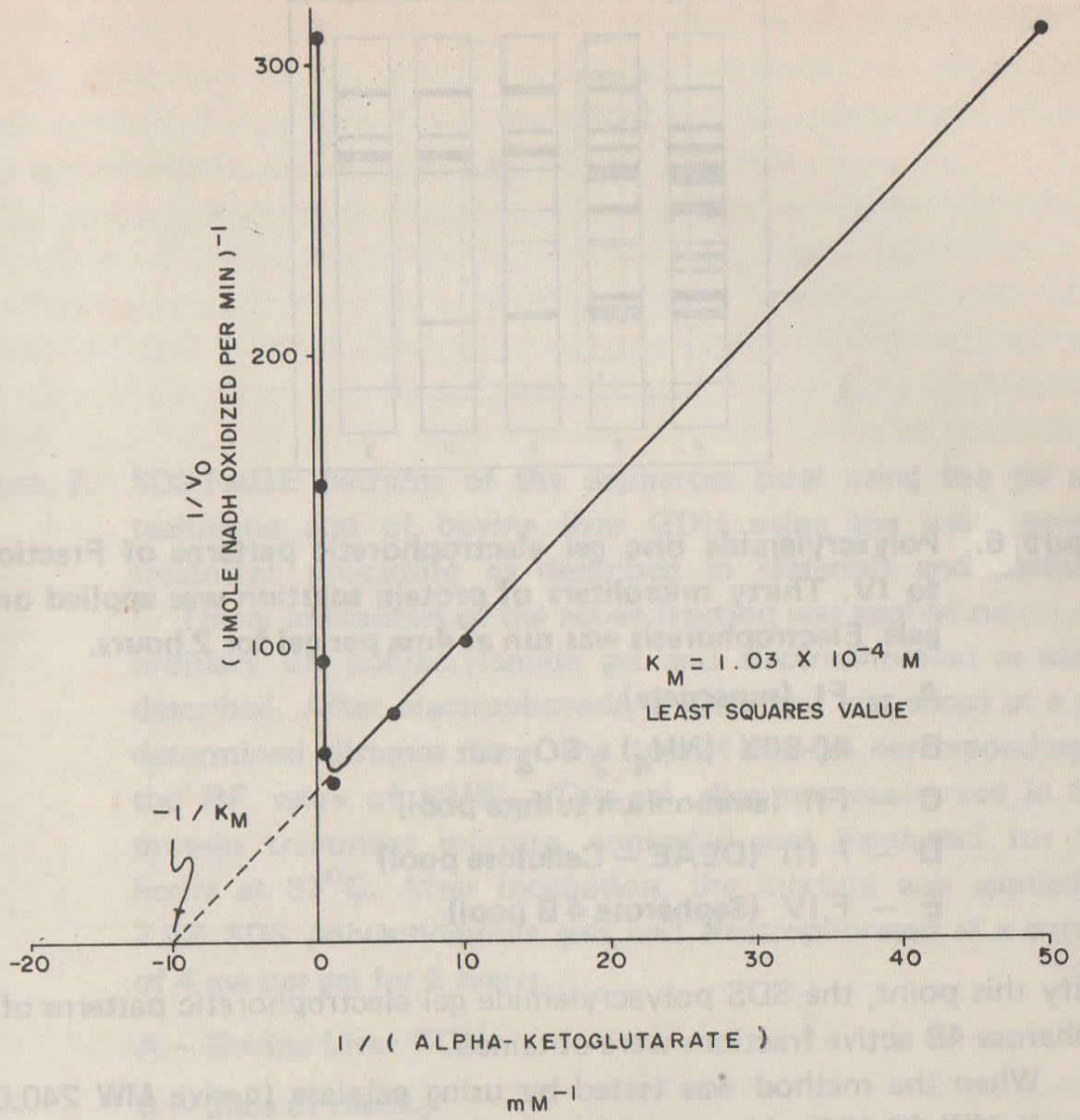

Figure 4. Lineweaver-Burk plot at varying concentrations of alpha-ketoglutarate. The plot is linear below 1 mM but shows substrate inhibition at higher concentrations. Linearity was assumed in extrapolating the plot to obtain the apparent Km value.

Electrophoretic protein patterns in polyacrylamide gels

Figure 5 shows the gel electrophoretic protein patterns of Fractions I to IV. It can be seen that although 95% of impurities has been removed, Fraction III is still a largely impure preparation. Fraction IV contains 4 major high molecular weight components (referred to here as HMW proteins). Although peaks from the Sepharose 4B column are well separated, the poor resolution is reflected by the appearance of impurities in the active fractions. However, it is clear that HMW_3 is the major protein band in the active fraction.

To ascertain the protein band corresponding to the GDH enzyme, the gels were stained with NBT/PMS to visualize the enzyme band as in Figure 6. After inspection, it appears that HMW_3 is the protein that best fits the specific activity pattern for the enzyme. However, HMW_3 may be a mixture of proteins having very close molecular weights and ionic charge. To

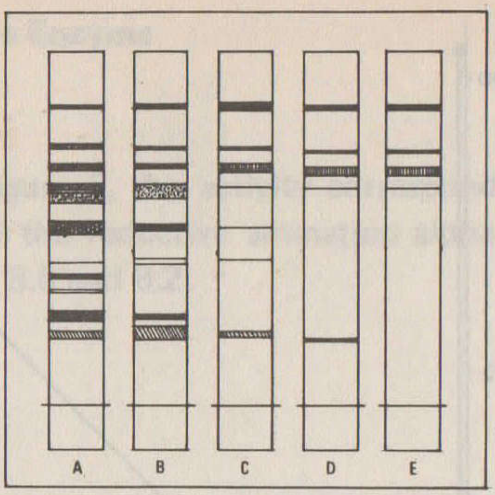

Figure 5. Polyacrylamide disc gel electrophoretic patterns of Fractions I to IV. Thirty microliters of protein solution was applied on 6% gels. Electrophoresis was run at 4ma per gel for 2 hours.

A – FI (supernate)
B – 40-60% $(NH_4)_2SO_4$ cut
C – FII (ammonium sulfate pool)
D – F III (DEAE – Cellulose pool)
E – F IV (Sepharose 4 B pool)

verify this point, the SDS polyacrylamide gel electrophoretic patterns of HMW$_3$ from Sepharose 4B active fractions were obtained.

When the method was tested by using catalase (native MW 240,000; subunit MW 60,000), identical SDS gel patterns were obtained for the gel slice compared with gels prepared using the usual procedure for protein treatment.

The data is shown in Figure 7. The SDS gel pattern of HMW$_3$ from fraction 20-21 shows 4 bands designated S_1, S_2, S_3 and S_4. From a plot of the logarithm of molecular weight of protein standards against the mobility in Figure 8, the molecular weights of the subunits were determined as shown in Table II.

The results indicate that, at this point, the purity of HMW_3 cannot be established. Although HMW_3 appears to be a single band, there is a good chance for a number of different proteins of the same ionic charge and very similar molecular size to be present. The GDH enzyme can just be one of those proteins.

DISCUSSION

The partial purification and characterization of prawn muscle glutamate dehydrogenase was achieved. The results demonstrate that *P. monodon* possesses an enzyme which catalyzes the reductive amination of alpha-ketoglutarate. As in other animal systems, it is probably the major enzyme responsible for the redistribution of alpha amino groups from amino acids

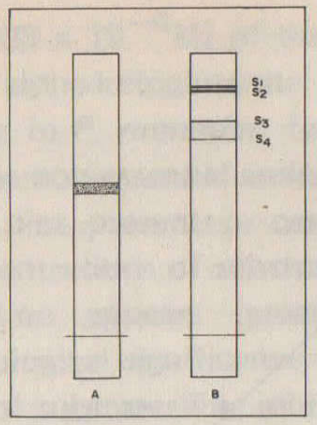

Figure 7. SDS-PAGE patterns of the Sepharose pool using the gel slice technique and of bovine liver GDH using the usual protein treatment procedure as described in Materials and Methods.

Thirty microliters of the active fraction was applied on ordinary 6% polyacrylamide gel and electrophoresed as earlier described. After electrophoresis, a 4mm gel was sliced at a predetermined distance from the top of the gel corresponding to the RF value of HMW_3. This gel slice was macerated in SDS protein treatment mixture, sonicated and incubated for two hours at 37°C. After incubation, the mixture was applied to 7.5% SDS polyacrylamide gels and electrophoresed at a current of 4 ma per gel for 6 hours.

A – Bovine Liver GDH

B – Slice of HMW_3

present in excess to compensate for those which are present in limited quantities.

Partial purification was attained employing a number of modifications into previously published procedure (5). A 260-fold purification was obtained, but the recovery of enzyme activity was found to be low. Dialysis of the active ammonium sulfate fractions overnight against Tris-chloride-EDTA buffer decrease the enzyme activity appreciably. In addition, standing the enzyme in buffer prior to gel filtration also decreased the activity. Furthermore, resolution on the Sepharose 4B column was found to be low as shown by the electrophoretic patterns in Figure 5, although a good separation was obtained. The extent of purification was, therefore, computed on the basis of the amount of protein recovered in the peak fraction since GDH activity was low.

Glutamate dehydrogenase has been isolated and purified from various sources (5, 6, 7, 13). It appears that the enzyme catalyzing the biosynthesis of glutamate is widely distributed. The prawn muscle enzyme demonstrates an optimum pH for the reductive amination of alpha-ketoglutarate between pH 8.0 to 8.2. It compares well with the bovine liver enzyme (13), the frog liver and the dogfish liver enzymes (4, 5).

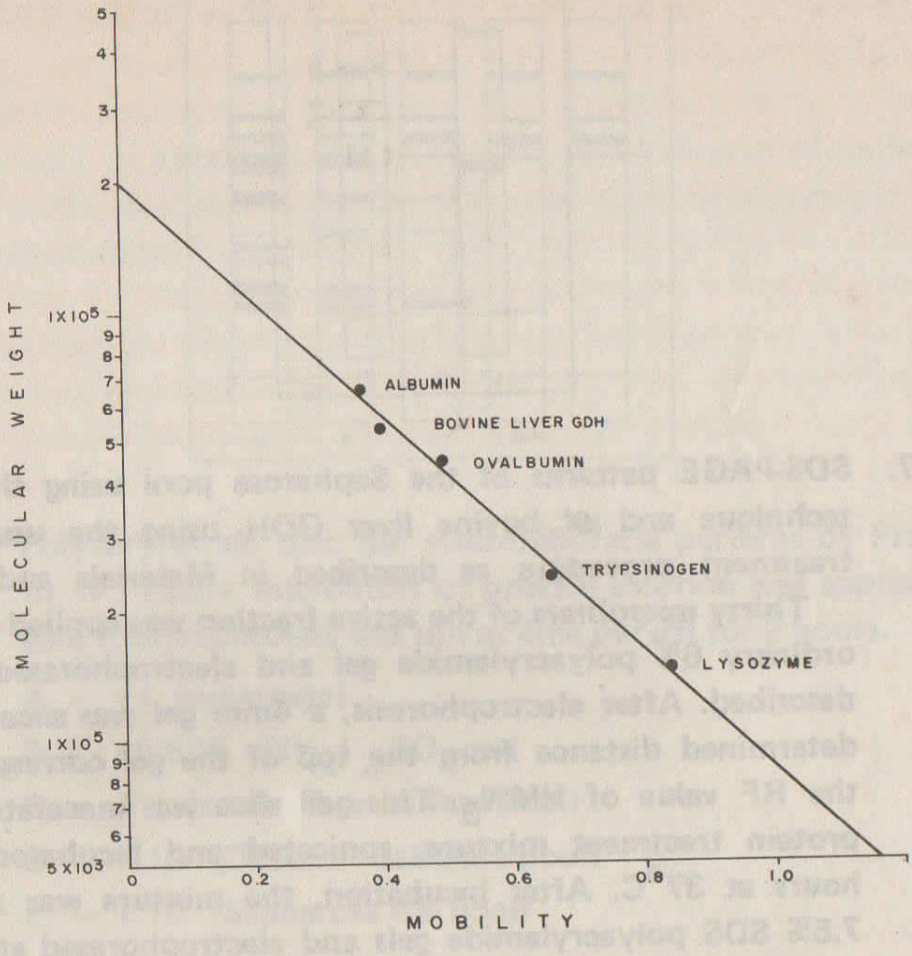

Figure 8. Plot of log MW against mobility to determine subunit molecular weight by SDS -PAGE.

Table 2. Subunit molecular weight determination by SDS-PAGE

Protein Subunit	Molecular Weight	Mobility	Average Mobility
Major bands			
S_1	141,000	0.118 0.116	0.117
S_4	88,000	0.265 0.261	0.263
Minor bands			
S_2	134,000	0.132 0.130	0.131
S_3	107,000	0.206 0.203	0.204

The low K_m value (1.03×10^{-4}M) of the enzyme in the presence of varying concentrations of alpha-ketoglutarate suggests the physiological significance of this pathway in *P. monodon* for the biosynthesis of glutamate and ultimately, of all the non-essential amino acids.

The Lineweaver-Burk plot presents a case of substrate inhibition at high, non-physiological concentrations of substrate. The concentration range at which substrate inhibition appears (greater than 1 mM of alpha-ketoglutarate) has no physiological significance except for setting the upper limits for the *in vivo* levels of subtrate. The inhibition may be a reflection of dead-end combination of the substrate with the form of the enzyme it is not supposed to bind with.

Rife and Cleland, 1980 (14) reported and characterized substrate inhibition by alpha-ketoglutarate on bovine liver glutamate dehydrogenase. Since $NADP^+$ enhance this inhibition, it was postulated to result from an abortive E-$NADP^+$–ketoglutarate complex.

The native molecular weight of the prawn muscle enzyme was determined from gel filtration to be about 320,000 daltons. Bovine liver GDH has a molecular weight of 270,000 daltons from molecular sieve and ultracentrifuge data (7). However, recently this has been reported to be about 313,000 daltons (15). On the other hand, the dogfish liver enzyme has a molecular weight of 330,000 daltons (5). It appears that the active subunit of the enzyme from these various sources are equivalent in terms of molecular size.

The protein pattern of the active fractions from gel filtration was obtained using polyacrylamide disc gel electrophoresis and sodium dodecyl sulfate (SDS) polyacrylamide disc gel electrophoresis. The results indicate that, at this point, the purity of the protein band corresponding to the GDH enzyme (HMW_3 protein) cannot be established. A number of different proteins with the same ionic charge and very similar molecular size may comprise this single band. The GDH enzyme could just be one of them.

The gel slice technique and the SDS-polyacrylamide gel electrophoresis were employed to study the subunit composition of the active protein band. Four proteins designated as S_1, S_2, S_3, and S_4 having molecular weights of 141,000, 134,000, 107,000, and 88,000, respectively, were obtained. The relative impurity of the active band limits the conclusions one can derive from the data.

With these considerations, one can only draw some possibilities regarding the subunit nature of the GDH enzyme.

Possible combinations that could comprise the holoenzyme were made from the molecular weights of S_1, S_2, S_3, and S_4. The model that appears to be consistent with all the experimental observations demonstrates an enzyme composed of one big and two small subunits (estimated MW of holoenzyme – 317,000 daltons). However, the possibility that four small subunits, a tetramer of S_4 proteins (estimated MW of holoenzyme – 352,000 daltons), comprise the holoenzyme cannot be disregarded.

This finding should remain preliminary until an entirely pure preparation of the prawn muscle GDH is obtained. They should only be taken as a guide until refinements on the gel filtration technique to solve the problem of poor resolution is made and until further purification of HMW_3 protein is done. A possible technique that can be used to further purify the enzyme is gel isoelectrofocusing. This will require a larger amount of starting material so that recovery is not sacrificed for retaining activity. In addition, other chromatographic techniques must be explored.

In comparing the prawn muscle glutamate dehydrogenase with the bovine liver enzyme, it is clear that the former has larger subunits than the latter (subunit MW 53,000). Moreover, there are indications that their respective subunit organizations are different. These findings may have evolutionary significance and help to explain the subtle differences in the character of these enzymes.

SUMMARY

The partial purification and characterization of L-glutamate dehydrogenase, a key enzyme in the biosynthesis of non-essential amino acids, from the muscle of *P. monodon* juveniles was achieved. Enzyme extraction with Tris-acetate buffer, ammonium sulfate fractionation, DEAE-Cellulose chromatography, and Sepharose 4B chromatography were used. A 260-fold purification was obtained, but the recovery of enzyme activity was low.

P. monodon possesses an enzyme which facilitates the biosynthesis of glutamate from alpha-ketoglutarate and ammonia. Its optimum pH for the reductive amination of alpha-ketoglutarate is between pH 8.0 and 8.2. The low Km value (1.03×10^{-4} M) of the enzyme for alpha-ketoglutarate strongly suggests the physiological significance of this pathway in *P. monodon*. Inhibition by alpha-ketoglutarate at high, non-physiological levels was noted.

The native enzyme has a molecular weight of 320,000 daltons from gel filtration. Subunit studies using SDS-polyacrylamide gel electrophoresis suggest that the enzyme possesses a unique subunit organization compared to those obtained from other sources.

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LITERATURE

1. Subramanian, S. *Biophys. Chem.* 7: 375-378 (1978).

2. Lehninger, A. L. *Biochemistry.* Worth Publishers, Inc., N.Y. (1975).

3. Eisenberg, H., R. Josephs and E. Reisler. *Adv. Prot. Chem. 30:* 101-181 (1976).

4. Fahein, L. A. and P. P. Cohen. *Methods of Enzymology.* (Tabor and Tabor, eds.) *43* : 839-844 (1970).

5. Corman, L. and A. Inamdar. *Methods of Enzymology.* Tabor and Tabor, eds.) *43:* 844-850 (1970).

6. Doherty, D. *Methods of Enzymology.* (Tabor and Tabor, eds.) *43:* 850-855 (1970).

7. Rogers, K. S., *et al. J. Biol. Chem. 240:* 198-200 (1965).

8. Frieden, C. *J. Biol Chem. 240:* 2028-2035 (1965).

9. Huggins, A. K. and K. A. Munday. *Adv. Comp. Physiol. Biochem.* (O. Lowenstein, ed.) Academic Press, N. Y. *3*: 271-378 (1968).

10. Lowry, O., *et al. J. Biol. Chem. 193:* 265-275 (1951).

11. Gabriel, O. *Methods of Enzymology.* (S.P. Colowick and N. O. Kaplan, eds.). 22: 578-604 (1971).

12. Weber, K. and M. Osborn, *J. Biol. Chem. 244:* 4406-4412 (1969).

13. Strecker, H. J. *Methods of Enzymology.* (S. P. Colowick and N.O. Kaplan, eds.). 2: 220-225 (1959).

14. Rife, J. E. and W. W. Cleland. *Biochemistry. 19:* 2321-2328 (1980).

15. Eisenberg, H. and G. M. Tomkins. *J. Mol. Biol. 31:* 37-49 (1968).