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Purification and Properties of Glutamate-phenylpyruvate Aminotransferase from Rumen Bacterium *Prevotella albensis*

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Abstract: Glutamate-phenylpyruvate aminotransferase (GPAase) from a cell free extract of a rumen bacterium *Prevotella albensis* was purified 36-folds. The molecular weight of the GPAase was estimated to be 39.0 kDa by SDS-PAGE. The optimum pH of GPAase was 6.2 and the activity declined markedly above pH 8.5. GPAase was reactive over a wide range from pH 4.5 to 10.5. The maximum reaction velocity of GPAase was observed at a temperature of 50°C and at higher temperatures over 60°C, the activity declined. The GPAase was stable below 60°C. Most of the chemical agents and metal ions showed inhibition effects on GPAase activity.

Key words: Rumen bacteria, *Prevotella albensis*, glutamate-phenylpyruvate aminotransferase, purification, characteristics, inhibition effects.

Introduction

Different amino acids are synthesized from their corresponding precursors and also broken down at different rates and to different products by the rumen microorganisms. Although bacteria play a significant role in the digestion and metabolism of feed in the rumen, information concerning the physiology and interactions with other ruminal microorganisms is scarce. Safely manipulation of the ruminal ecosystem in order to improve ruminal metabolism is still technically difficult because the physiology, metabolism and metabolic interactions of the ruminal microorganisms is not completely understood. Molecular techniques must continue to be used to determine the fundamental details of how ruminal microorganisms and their enzymes carry out digestion and fermentation. From an enzymatic perspective, information on the structure and mode of action of proteins which act in an anaerobic environment and are originated from a poorly studied prokaryotic microorganism will be of general biochemical interest and the possibilities to use them in medical or industrial applications deserve to be explored.

Phenylalanine (Phe) is one of the aromatic amino acids and is thought to be essential for ruminant animals. The synthesis of Phe through the aminotransferase of phenylpyruvic acid (PPY) by mixed rumen bacteria and protozoa has recently been demonstrated by Amin and Onodera (1997), and widely in aerobic microoganisms such as *E. coli* (Gelfand and Steinberg, 1977; Powell and Morrison, 1978, and Calton *et al.*, 1986), *Bacillus subtilis* (Mavrides and Comerton, 1978), *Corynebacterium glutamicum* (Bulot and Cooney, 1985), *Pseudomonas fluorescens* (Evans *et al.*, 1987), and *Pseudomonas putida* (Ziehr *et al.*, 1984; Ziehr and Kula, 1985). The aminotransferase activity in the rumen has already been demonstrated by some investigators where phenylalanine aminotransferase activity by mixed rumen bacteria and protozoa has also been reported (Tsubota and Hoshino, 1969, and Bhatia *et al.*, 1979, 1980), but the enzymes from the pure cultures are not yet purified and there have been no report concerning the glutamate-phenylpyruvate aminotransferase (GPAase)(EC 2. 6. 1. 64) of rumen bacteria.

This study was conducted to purify and investigate some characteristics of GPAase from rumen bacterium *Prevotella albensis*.

Materials and Methods

The study was carried out in the laboratory of Animal Nutrition and Biochemistry, Division of Animal Science, Faculty of Agriculture, Miyazaki University, Japan.

Chemicals: Phenylpyruvic acid sodium salt was purchased from Tokyo Kasei (Tokyo, Japan). Sodium phosphate (monobasic and dibasic), ammonium chloride, sodium L-glutamic acid (monohydrate), NADH, potassium phosphate (monobasic and dibasic), ammonium sulfate, sodium chloride, sodium lactate, sodium bicarbonate, magnesium sulfate, calcium chloride (anhydrous), aminooxyacetic acid, 2-mercaptoethanol (ME), resazurin sodium salt, mercury chloride and D-glucose (anhydrous) were from Wako Pure Chemical Industries (Osaka, Japan). Bacto-casitone and yeast extract were from Difco Laboratories (Michigan, USA). Cellobiose, Phenylhydrazine, 1,10-phenantrolin, semicarbazide, kynuric acid, 2, 2-bipyridyl, 4-(hydroxymercuri) benzoic acid, D-cycloserine, sodium dodecyl sulphate (SDS), EDTA, $AgNO_3$, NaN_3, $CuSO_4$, $CuCl_2$, $HgCl_2$, $SnCl_2$, $MgCl_2$, $BaCl_2$, $CaCl_2$, $MnCl_2$, $SrCl_2$, KCl and pyridoxal-5-phosphate (PLP) were obtained from Nacalai Tesque (Kyoto, Japan), and L-cystein and maltose (monohydrate) were from Kanto Chemicals (Tokyo, Japan). Glutamate dehydrogenase (EC 1.4.1.2) (GDH) was purchased from Toyobo Chemicals (Osaka, Japan). Phenyl-superose, DEAE-toyopearl 650M, Sephacryl S-100 HR were obtained from Pharmacia (Uppsala, Sweden).

Preparation of culture media: Media 2 (Hobson, 1969) were used to grow the bacterial cells. The successful growth of the rumen bacterium relies on the maintenance of anaerobic conditions at all times. This is achieved through a combination of bubbling all media components with O_2 free CO_2 by passing the gas over copper turning heated to 350°C and the chemical reducing agent cysteine.

Medium was boiled twice to remove oxygen, cysteine was added and the medium was boiled again and then allowed to cool to 50°C, while bubbling with O_2 free CO_2. About 350 ml of medium was dispensed into 500 ml wheaton bottle including non toxic butyl rubber stopper and a screw cap taking care to maintain a constant stream of O_2 free CO_2 through both the main bulk of medium and that in the bottles. The bottle was sealed with butyl rubber stopper and screw

cap and autoclaved.

Bacterial strain and inoculation: The anaerobic rumen bacterium *Prevotella albensis* was used in present study. *P. albensis* was supplied by Dr. R. J. Wallace, Rowett Research Institute, Scotland. Inoculation was carried out at room temperature with sterilized syringe and needle through stopper. Cells were grown overnight at 39°C.

Preparation of the cell extract: Culture media was centrifuged at 27000xg for 15 min at 4°C to get bacterial cells. The cells were washed once in 25 mM (pH 6.2) anaerobic phosphate buffer containing 0.1 mM PLP and 0.01% ME. Centrifuged again at 27000xg for 15 min at 4°C and resuspended in 30 ml of the same buffer. The cells were disrupted by sonication and the supernatant was collected by centrifugation at 37000xg for 45 min at 4°C and used as crude enzyme.

Purification of GPAase:

Precipitation and dialysis: Ammonium sulfate was added and mixed with crude enzyme to give 80% saturation and left to settle for an hour. The precipitates were collected by centrifugation at 37000xg for 15 min and dissolved in 0.01 mM Na-phosphate buffer (pH 6.2) containing 0.1 mM PLP and 0.01% ME. The enzyme solution was dialyzed overnight at 4°C with same buffer and collected by centrifugation at 37000xg for 15 min.

Phenyl-superose column chromatography: The dialyzate was first applied to a column of phenyl-superose (1.6 x 40 cm^2), pre-equilibrated with 0.01 mM Na-phosphate buffer (pH 6.2) containing 0.1 mM PLP and 0.01% ME. Protein was eluted with a linear concentration gradient (0.02 to 0.2 M) Na-phosphate buffer of pH 6.2 containing 0.1 mM PLP and 0.01% ME.

DEAE-toyopearl 650M column chromatography: The active fractions from the first column were applied to a DEAE-toyopearl 650M column (1.6 x 40 cm^2). The column was washed with 0.01 mM Na-phosphate buffer (pH 6.2) containing 0.1 mM PLP and 0.01% ME and eluted with a linear concentration gradient NaCl (0.05 to 0.5 M) with the same buffer.

Sephacryl S-100 HR gel filtration: The active fractions from the second column were applied to a sephacryl S-100 HR gel filtration column (2.6 x 100 cm^2), pre-equilibrated with 0.01 mM Na-phosphate buffer (pH 6.2) containing 0.1 mM PLP and 0.01% ME and eluted with the same buffer.

Aminotransferase assay: The reaction mixture was composed of 0.1 M sodium phosphate buffer (pH 6.2), 0.5 mM PLP, 10 mM ammonium chloride, 15 mM L-glutamic acid sodium salt, 5 mM phenylpyruvic acid sodium salt, 2.6 units GDH, 2 mM NADH and transaminase enzyme extract in a total volume of 200 μl. The reaction was initiated by addition of the enzyme extract to the mixture and incubated at 50°C for 20 min. The 2-ketoglutarate formed by transamination, was converted to 2-ketobutyrate by reductive decarboxylation. This conversion is directly coupled with NADH consumption which was measured by the change in absorption at 340 nm. For photometric assays a micro plate reader (Labsystems Multiskan MS, version 8.0, Helsinki, Finland) was used. For calculation of the enzyme activity a linear correlation equation with different concentration of NADH was used and the differences of NADH oxidized with and without substrate (PPY) were measured and the production of phenylalanine was calculated. The substrate-amino donor ratio was chosen as 1:3, demonstrated by Evans *et al.* (1987).

Protein measurement: Protein was monitored by the measurement of absorbance at 280 nm during purification. The protein concentration of the enzyme was measured by the method of Bradford (1976), using bovine serum albumin as a standard.

Characterization of GPAase

Molecular size estimation: The molecular size of the GPAase of *Prevotella albensis* was determined by sodiumdodecyl sulphate polyacryl amide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). The GPAase was run through a native gel (omitting SDS and 2-ME) at 4°C and the band was identified by means of coupled reaction catalyzed by GDH and diaphorase, leading to the reduction of iodonitro tetrazolium chloride (INT) to a coloured formazan (Bergmeyer, 1987). Bands of activity appeared and visually inspected as pink areas on a clear background. The band was cut off, treated with SDS and 2-ME and run through SDS-PAGE.

Following reaction sequence was observed:

$$\text{L-Phe} + \text{2-oxoglutarate} \xrightleftharpoons{\text{PheAt}} \text{L-Glu} + \text{PPY}$$

$$\text{L-Glu} + NAD^+ + H_2O \xrightleftharpoons{\text{GDH}} \text{2-oxoglutarate} + NADH + NH_4^+$$

$$NADH + INT + H^+ \xrightarrow{\text{diaphorase}} NAD^+ + \text{forazan}$$

The gels were stained for proteins with Coomassie brilliant blue G-250 or silver stain Wako kit. Marker proteins used were rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45.0 kDa), bovine carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5) and hen egg white lysozyme (14.4 kDa). Molecular mass of the purified enzyme was calibrated from the mobility of the enzyme protein with those of standard proteins determined by SDS-PAGE.

Effect of pH on the activity and stability of GPAase: The GPAase activity of *Prevotella albensis* was assayed at different pH values from 4.0 to 10.5 under standard conditions. The enzyme was also stored at different pH values as mentioned above for 15 min at 4°C, and the remaining activities were assayed.

Effect of temperature on the activity and stability of GPAase: Activity and stability of GAPase was measured at various temperatures from 25-70°C at pH 6.2 under standard conditions. Thermal stability of GPAase was also investigated by determining the residual activity after keeping the enzyme at various temperatures as mentioned above for 15 min.

Effects of chemical agents and metal ions on the activity of GPAase: GPAase activity of the enzyme was measured with chemical agents and metal ions. The enzyme was pre-incubated with the components to be tested at the concentrations of 10.0, 1.0 and 0.1 mM for 20 min at 50°C. The relative activity was determined by the standard assay system as described earlier.

Results and Discussion

NADH concentration: A linear relationship was observed between the absorbency and different NADH concentrations

Table 1: Purification of glutamate-phenylpyruvate aminotransferase (GPAase) of rumen bacterium *Prevotella albensis*.

Fractions	Total protein (mg)	Total activity (μM)	Specific activity (μM/mg)	Yield%	Fold
Crude enzyme Ammonium	9680	3992	0.41	100	1.0
Sulphate (80%)	4131	2593	0.63	65.0	1.5
Phenyl-superose	1242	1576	1.27	39.5	3.1
DEAE toyopearl 650M	174	683	3.93	17.1	9.6
Sephacryl S-100 HR	33	484	14.67	12.1	35.8

Table 2: Effects of chemical agents and metal ions on the activity of Purified glutamate-phenylpyruvate aminotransferase (GPAase) of rumen bacterium *Prevotella albensis*.

Component	Relative activity %		
	Component concentrations (mM)		
	10.0	1.0	0.1
None	100.0	100.0	100.0
Aminooxyacetic acid	-	0	0
Phenylhydrazine	-	0	0
Semicarbazide	-	0	15.0
Sodium dodecyl sulfate	0	0	58.0
4-(hydroxymercuri) benzoic acid	-	0	99.0
EDTA	*	22.0	-
1, 10-phenantrolin	101.0	-	-
2, 2-bipyridyl	98.0	-	-
Kynuric acid	0	100.0	-
D-cycloserine	-	-	80.0
$AgNO_3$	*	0	101.0
NaN_3	91.0	-	-
$CuSO_4$	*	0	102.0
$CuCl_2$	*	0	100.0
$HgCl_2$	*	*	99.0
$SnCl_2$	*	0	100.0
$MgCl_2$	98.0	-	-
$BaCl_2$	*	101.0	-
$CaCl_2$	84.0	-	-
$MnCl_2$	*	102.0	-
KCl	78.0	-	-
$SrCl_2$	72.0	-	-

The glutamate-phenylpyruvate aminotransferase (GPAase) activity of the enzyme was measured with chemical agents and metal ions in 0.01mM Na-phosphate buffer (pH 6.2) containing 0.1mM PLP and 0.01% 2-ME. The enzyme was pre-incubated with the component to be tested at the given concentrations for 20 min at 50°C. The relative activities were expressed as percentage activity measured with chemical reagents and metal ions to those in the absence of those chemical compounds.
-, not tested; *, the reaction mixture became milky and showed abnormal reading.

by microplate reader. Thus the concentration of NADH could readily be calculated from absorbency. NADH concentration from 0.5 mM to the lowest showed high linearity (Fig. 1).

Purification of GPAase: The purification steps of GPAase are illustrated in Table 1. Most of the GPAase was found in the protein fraction precipitated by ammonium sulphate at 80% saturation. Only one GPAase active peak was observed in all the column chromatograms. The GPAase was finally purified 35.8-folds by the three steps of process but the recovery was about 12.1% (Table 1). The molecular weight of the GPAase was estimated to be 39.0 kDa by SDS-PAGE (Fig. 5).
There is no report to compare concerning the GPAase of the rumen bacterium *Prevotella albensis*. The molecular size of aspartate-phenylpyruvate aminotransferase from *Pseudomonas putida* (Zeihr and Kula, 1985) have been reported to be 72.0 kDa and composed of two subunits of 36.0 kDa each and of *E. coli* (Powell and Morrison, 1978) was 90.0 kDa.

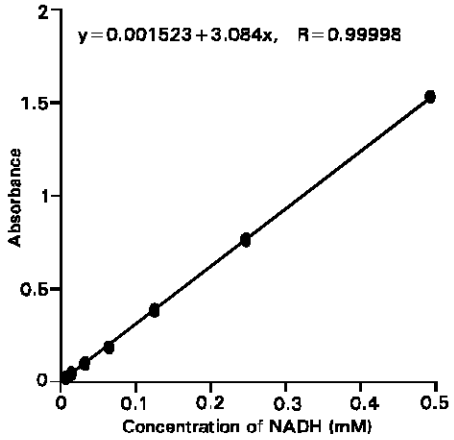

Fig. 1: Linear relationship between different concentrations of NADH (mM) and absorbance by microplate reader.

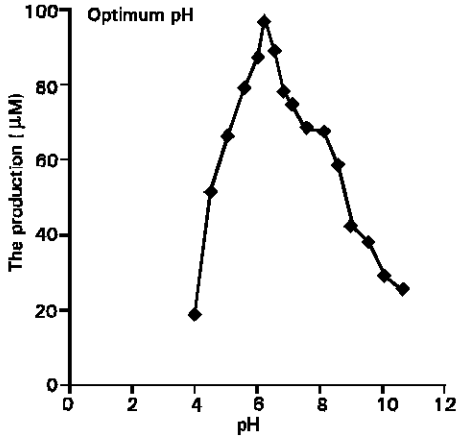

Fig. 2: Effect of pH on the activity of GPAase from *Prevotella albensis*.

Characteristics of GPAase: In the present study, PLP and ME were used to increase the activity and stability of the aminotransferase (Powell and Morrison, 1978; Ziehr and Kula, 1985). The stability of the enzyme was affected by different pH and temperatures.

Effect of pH: The optimum pH of GPAase of *P. albensis* was found to be 6.2 (Fig. 2). This value was lower than the values of 10.0 of *Pseudomonas putida* (Zeihr and Kula, 1985), 12 of *Pseudomonas fluorescens* (Evans *et al.*, 1987), and 7.2-7.5 and 9.0 of *E. coli* (Powell and Morrison, 1978; Calton *et al.*, 1986, respectively) for aspartate-phenylpyruvate aminotransferase activity.

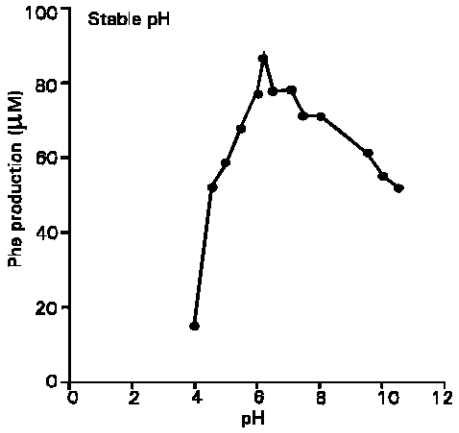

Fig. 3: Effect of pH on the stability of GPAase from *Prevotella albensis*.

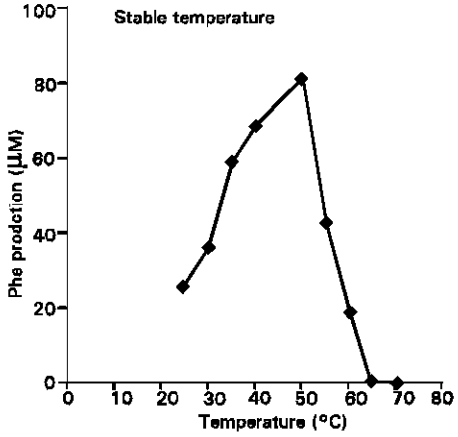

Fig. 4: Effect of temperature on the activity and stability of GPAase from *Prevotella albensis*.

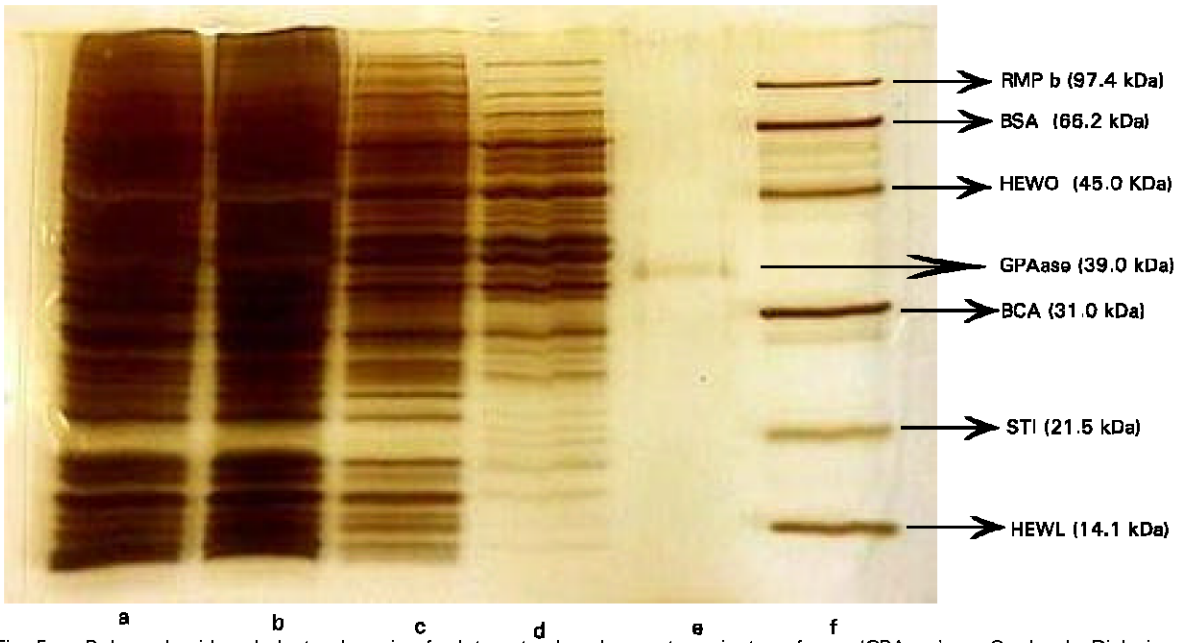

Fig. 5: Polyacrylamide gel electrophoresis of glutamate-phenylpyruvate aminotransferase (GPAase). a. Crude, b. Dialysis, c. 1st column d. 2nd column, e. 3rd column and f. Standard protein.

Where, RMP b: Rabbit muscle phosphorylase b
BSA: Bovine serum albumin
HEWO: Hen egg white ovalbumin
GPAase: Glutamate-phenylpyruvate aminotransferase
BCA: Bovine carbonic anhydrase
STI: Soybean trypsin inhibitor
HEWL: Hen egg white lysozyme.

In present study, GPAase of *P. albensis* was reactive over a wide range from pH 4.5 to 10.5 (Fig. 3) but the activity declined markedly above pH 8.0, whereas aspartate-phenylpyruvate aminotransferase from *Pseudomonas putida* and *E. coli* showed reactivity from 6.0 to 11.5 and 6.0 to 9.0 and declined above pH 10.0 and 9.0 as reported by Zeihr and Kula (1985) and Calton *et al.* (1986), respectively.

Effect of temperature: The maximum reaction velocity of GPAase of *P. albensis* was observed at a temperature of 50°C and the activity was markedly declined at higher temperatures over 60°C (Fig. 4). On the other hand, Zeihr and Kula (1985) demonstrated maximal reaction velocity of aspartate-phenylpyruvate aminotransferase from *Pseudomonas putida* at a temperature of 55°C and declined above 55°C. The optimum temperature from *Pseudomonas fluorescens* was demonstrated to be 37°C (Evans *et al.*, 1987).

Inhibition effects: The effects of chemical agents and metal ions on the activity of purified GPAase of *P. albensis* are mentioned in Table 2. The GPAase activity was completely inhibited by the use of aminooxyacetic acid, an

aminotransferase inhibitor (Miflin and Lee, 1975), and phenylhydrazine. Semicarbazide, SDS and D-cycloserine were very strong inhibitors even in low concentration, whereas 4-hydroxymercuri benzoic acid, $AgNO_3$, $CuSO_4$, $CuCl_2$, $HgCl_2$, $SnCl_2$, EDTA, $BaCl_2$, $MnCl_2$ and kynuric acid were strong with high but had no inhibition effect with low concentrations. The inhibition of sodium azide, $SrCl_2$, KCl, and $CaCl_2$ were weaker even in high concentration. There was no inhibition effect by $MgCl_2$, 1, 10-phenantrolin, and 2, 2-bipyridyl. In this connection, Zeihr and Kula (1985) demonstrated the effects of 14 inhibitors on aspartate-phenylpyruvate aminotransferase from *Pseudomonas putida* where 12 inhibitors are common with the present experiment. The relative activities were more or less close with the present findings except EDTA and $CuSO_4$ that had no effect with high concentration.

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