

## Isolation, purification, and characterization of L-glutamate oxidase from *Streptomyces* sp. 18G

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**Abbreviations:** GDC: Glutamate decarboxylase  
GDH: Glutamate dehydrogenase;  
GLOD: L-glutamate oxidase.

**An extracellular L-glutamate oxidase (GLOD) was purified from soil-isolated *Streptomyces* sp 18G. The enzyme had a molecular weight of approximately 120,000 and consisted of two identical subunits, each with a molecular weight of 61,000. The isoelectric point was pH 8.5 and the enzyme had an optimal pH between 7.0–7.4. GLOD showed the maximum activity at 37°C. The GLOD activity was stable at pH ranging from 6.5 to 7.0 for 1 hr. Among 21 amino acids tested for substrate specificity, L-glutamate was almost exclusively oxidized. D-glutamate and L-aspartate were oxidized but only to extents of 0.79% and 0.53%, respectively.**

L-glutamate is an important amino acid widely used as a food additive because of its taste enhancing property. In neurochemistry, it is a major excitatory neurotransmitter of the central nervous system and the enteric nervous system (Cooper and Pritchard, 1994; Zilkha et al. 1995; Valero and García-Carmona, 1998). Based on this information, it is obviously very important to develop specific analytical

methods for measuring this amino acid, preferably in simple and reliable way (Valero et al. 1998).

L-glutamate can be measured by chromatographic methods (Kondrat et al. 2002; Hanco and Rohrer, 2004) which are complicated, time-consuming and require extensive sample pretreatment. The enzymatic method is chosen to overcome the problems mentioned above. Glutamate dehydrogenase (GDH) and glutamate decarboxylase (GDC) have been employed for the determination of L-glutamate (Shi and Stein, 1996; Liu et al. 1999; Ling, et al. 2000; Oliveira et al. 2001; Qhobosheane et al. 2004; Rodriguez et al. 2004). However, the GDC and GDH have some drawbacks due to poor substrate specificity and the requirement for expensive coenzyme such as NAD<sup>+</sup>. L-glutamate oxidase (GLOD) is used instead due to the relatively high substrate specificity comparing to GDH and GDC and no requirement for additional coenzyme. GLOD is an enzyme that specifically catalyzes the oxidative deamination of L-glutamate in the presence of water and oxygen with the formation of

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**Table 1. Purification of GLOD from *Streptomyces* sp. 18G.**

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg <sup>-1</sup> )	Yield (%)	Purification (fold)
Culture extract	1,203	185.25	0.15	100	1.0
Ammonium sulphate	376.6	151.03	0.4	81.53	2.6
SP-SepharoseFF	10.7	70.23	6.56	37.91	42.7
Q-SepharoseFF	0.72	33.43	46.48	18.05	302
Gel filtration	0.2	30.85	152.36	16.65	990

a-ketoglutarate, ammonia and hydrogen peroxide (Kusakabe et al. 1983; Böhmer et al. 1989; Fukunaga et al. 1998). The hydrogen peroxide formed in this reaction can easily be detected by the chromogenic peroxidase reaction or amperometric method (Böhmer et al. 1989; Villarta et al. 1991; Almeida and Mulchandani, 1993; Zilkha et al. 1995; Niwa et al. 1997; Chang et al. 2003). Therefore, L-glutamate oxidase holds excellent potential for use as the principle component in the determination of L-glutamate (Chen and Su, 1991; White et al. 1994; Ye et al. 1995; Matsumoto et al. 1998; Udomsopagit et al. 1998; Valero and Garcia-Carmona, 1998; Yao et al. 1998), although the presently available GLOD still has several disadvantages such as broad substrate specificity of the enzyme from some microorganisms (Kamei et al. 1983) and high cost (Kusakabe et al. 1983). In this study, we conducted a screening for glutamate oxidase-producing microorganisms from natural sources and investigating the physical and biochemical characteristics of the GLOD after the purification steps.

## MATERIALS AND METHODS

### Materials

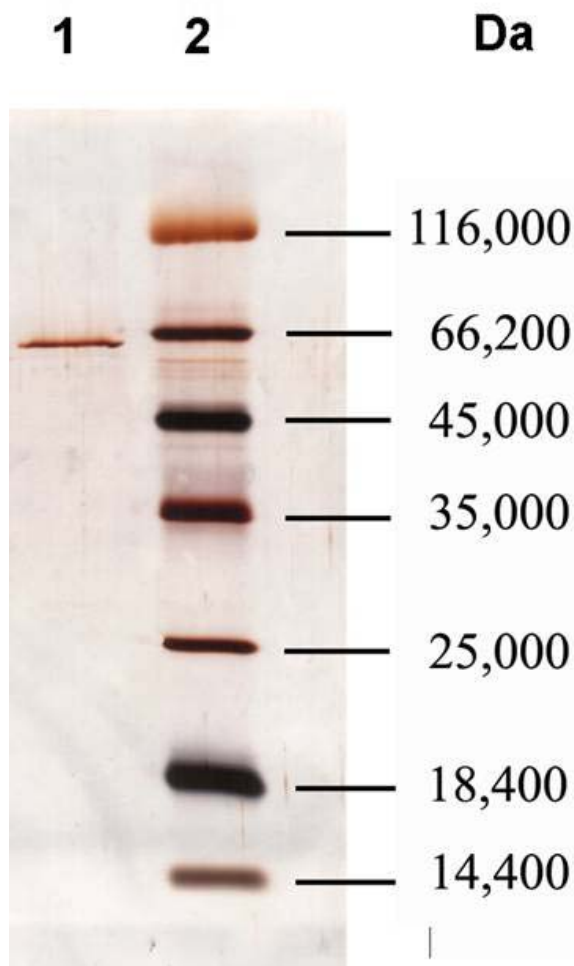
SP-Sepharose Fast Flow, Q-Sepharose Fast Flow, and Superdex 200 HR 10/30 were from Amersham Biosciences Ltd. (Uppsala, Sweden). Protein markers for gel filtration and amino acids were from Sigma-Aldrich Co. (St. Louis, USA).

### Microorganisms and culture conditions

*Streptomyces* sp. 18G was isolated from soil sample in Khlong Luang District, Pathum Thani Province, Thailand. The medium used for the screening was humic acid-vitamin agar (Hayakawa and Nonomura, 1987). The microorganism grown on inorganic salt starch agar medium (Williams et al. 1983) had spiral mycelia with spores. The colonies were tough, leathery and developed to powder and velvet colonies after spore forming. These characteristics suggested that the GLOD producing-microorganism was of the genus *Streptomyces*.

### Screening methods

The GLOD producing strain was selected by using the



**Figure 1. SDS-polyarylamide gel electrophoresis of GLOD from *Streptomyces* sp. 18G.** Lane 1, purified GLOD oxidase; lane 2, marker proteins, lysozyme (14,400),  $\beta$ -lactoglobulin (18,400), restriction endonuclease Bsp981 (25,000), lactate dehydrogenase (35,000), ovalbumin (45,000), bovine serum albumin (66,200) and  $\beta$ -galactosidase (116,000).

**Table 2. Substrate specificity of GLOD.** The enzyme activity was measured as described in text. All values represent the percentage activity compared with the value obtained from L-glutamate. UD = undetectable.

Substrate (10 $\mu$ M)	Relative activity (%)
L-glutamate	100
D-glutamate	0.79
L-glutamine	UD
L-aspartate	0.53
L-glycine	UD
L-alanine	UD
L-arginine	UD
L-cysteine	UD
L-histidine	UD
proline-L-hydroxy-4-trans	UD
lysine hydrochloride-L	UD
L-proline	UD
L-serine	UD
L-threonine	UD
L-valine	UD
L-isoleucine	UD
L-methionine	UD
L-phenylalanine	UD
L-leucine	UD
L-tryptophan	UD
L-tyrosine (1 $\mu$ M)	UD

method based on  $H_2O_2$ -dependent peroxidase catalyzed chromogenic reaction, as described by Li et al. 1996. Filter papers were dipped into the reaction mixture containing 2 U/ml horseradish peroxidase (HRP), 10  $\mu$  mole monosodium glutamate (MSG), 10  $\mu$  mole 4-aminoantipyrine (AAP) and 17.5  $\mu$  mole phenol in 0.1 M potassium phosphate buffer pH 7.4, prior to placing on the isolated colonies. The reaction was allowed to take place by incubating for 15-30 minutes at ambient temperature without light. GLOD producing colonies that turned the filter paper red were collected.

### GLOD production culture

*Streptomyces* sp. 18G was grown in wheat bran precultivation medium described by Böhmer et al. 1989 with some modifications. The medium contained 2.0% wheat bran, 0.5% sodium chloride and 0.5% monosodium glutamate. The culture was grown at 37°C with shaking at 200 rpm.

### GLOD assay

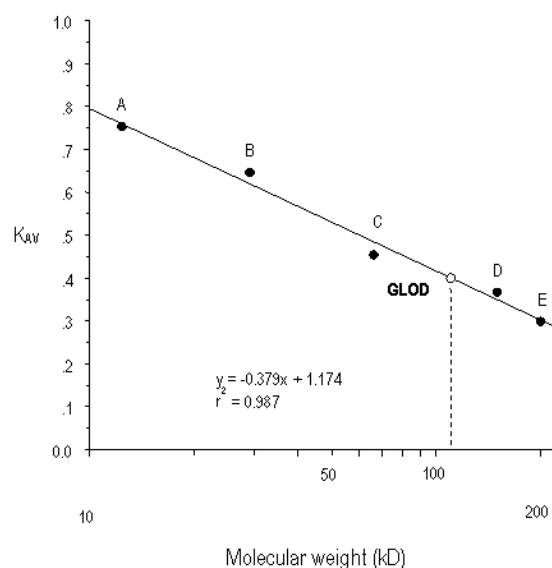
GLOD activity was assayed following the methods described by Li et al. 1996. The reaction mixture contained 1.0  $\mu$  mole 4-aminoantipyrine, 17.5  $\mu$  mole phenol, 2.5 U horseradish peroxidase and a sufficient amount of GLOD in 0.1 M potassium phosphate buffer pH 7.4 in a total volume of 1.30 ml. After a pre-incubation for 2 min at 37°C, the reaction was initiated by addition of 10  $\mu$  mole MSG to the reaction mixture. The absorbance at 500 nm was measured after incubated for 30 min at 37°C with gentle shaking. One unit of enzyme activity is defined as the amount of enzyme required to produce 1  $\mu$  mol of  $H_2O_2$  per minute under the assay conditions.

### Protein determination

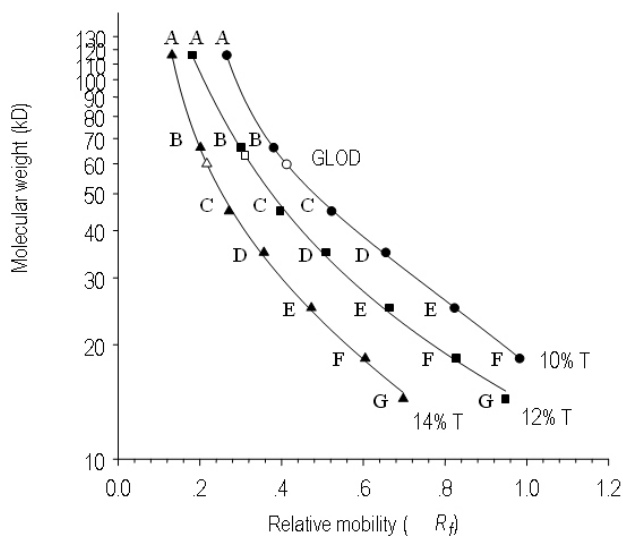
Protein was determined using Bradford assay. Bovine serum albumin (BSA) was used as a standard.

### Purification of GLOD

**Enzyme concentration.** Ammonium sulphate precipitation technique was used to concentrate the crude enzyme. Grounded ammonium sulphate was gradually added to the chilled enzyme solution while stirring until 80% saturation was obtained. The solution was then stirred at 0°C for 1 hr. The precipitate was collected by centrifugation at 10,000



**Figure 2. Molecular weight determination of native GLOD by Superdex 200 HR 10/30 gel filtration chromatography.** The molecular weight used were: A, cytochrome C (12,400); B, carbonic anhydrase (29,000); C, bovine serum albumin (66,000); D, alcohol dehydrogenase (150,000) and E,  $\beta$ -amylase (200,000).



**Figure 3. Estimation of molecular weights of GLOD subunits by SDS-PAGE in 3 different polyacrylamide separating gel concentrations, i.e. 10%, 12% and 14% with 4% stacking gel.** The molecular weight markers used were: A, lysozyme (14,400); B,  $\beta$ -lactoglobulin (18,400); C, restriction endonuclease *Bsp981* (25,000); D, lactate dehydrogenase (35,000); E, ovalbumin (45,000); F, bovine serum albumin (66,200) and G,  $\beta$ -galactosidase (116,000).

rpm, at 4°C for 45 min. The protein pellet was dissolved in a minimal volume of 20  $\mu$  M chilled potassium phosphate buffer pH 6.0. The enzyme solution was dialyzed overnight against the same buffer at 4°C.

**Cation-exchange chromatography.** The concentrated enzyme was applied to SP-Sepharose FF column with a bed volume of 30 ml. The column was pre-equilibrated with 20  $\mu$  M potassium phosphate buffer pH 6.0. After the column was washed with 40 ml of potassium phosphate buffer, the enzyme was eluted with a linear salt gradient of 0-3 M sodium chloride at a flow rate of 1 ml/min. Each fraction of the enzyme solution was tested for GLOD activity. The active fractions were pooled and concentrated by means of a centrifugal ultrafiltration and kept in an ice bath for further purification steps.

**Anion-exchange chromatography.** The pooled active fractions were desalted prior to loading into the pre-equilibrated Q-Sepharose Fast Flow column with a bed volume of 30 ml. The column was washed with 20  $\mu$  M Tris-HCl buffer pH 8.0 at the flow rate of 1 ml/min. Other proteins which bound to the column were then eluted by using linear salt gradient from 0.0-1.0 M sodium chloride at the same flow rate. The active fractions were pooled and concentrated by means of the centrifugal ultrafiltration with MW cut off at 10,000 and kept in an ice bath for the next step of enzyme purification.

**Gel filtration chromatography.** Gel filtration chromatography was used for the last step of enzyme purification. The Superdex 200 HR 10/30 column with 24 ml bed volume was pre-equilibrated with 50  $\mu$  M sodium

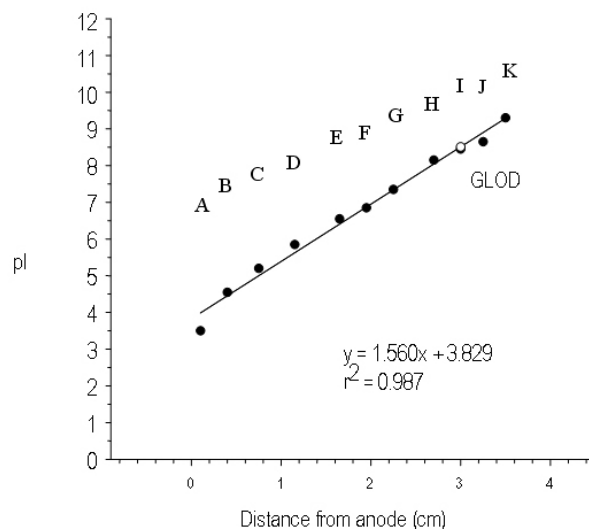
phosphate buffer pH 7.0 with 0.15 M sodium chloride. The concentrated enzyme from the previous step was applied. The enzyme was eluted from the column with the same buffer at a flow rate of 0.5 ml/min. Active fractions were determined for GLOD activity by the colorimetric method, and were pooled and concentrated for further assays.

### SDS-polyacrylamide gel electrophoresis

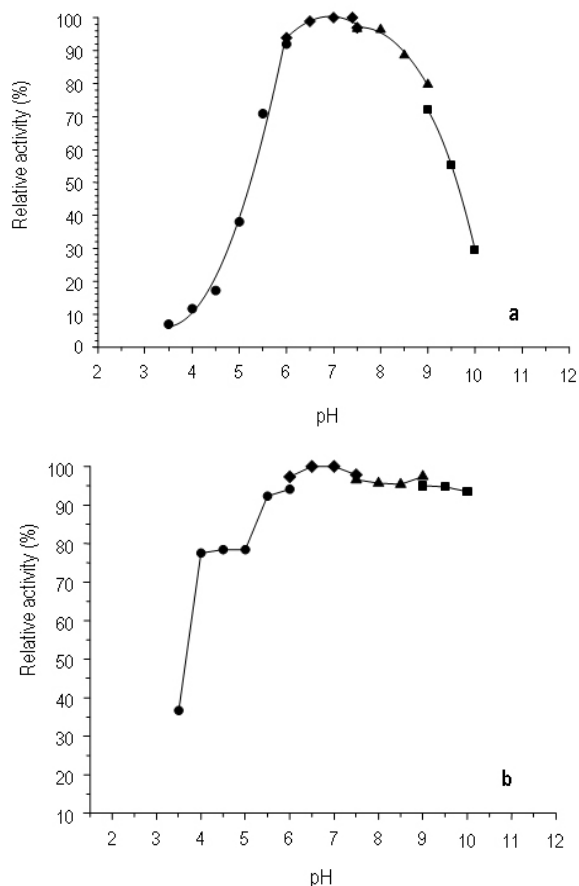
SDS-PAGE slab gel was carried out using a Mini-PROTEAN3<sup>®</sup> cell (Bio-Rad, Hercules, USA). Molecular weight markers were obtained from Sigma-Aldrich Corp., St. Louis, USA. The gels were subjected to protein bands visualization with silver staining method.

### Molecular weight determination by gel filtration chromatography

The relative molecular weight ( $M_r$ ) of the native enzyme was determined by using Superdex 200 HR 10/30 column. Elution was done at the flow rate of 0.25 ml/min with an elution buffer comprising 50  $\mu$  M sodium phosphate buffer pH 7.0 and 0.15 M NaCl. The calibration curve was constructed using protein markers: cytochrome C (12,400), carbonic anhydrase (29,000), bovine serum albumin (66,000), alcohol dehydrogenase (150,000) and  $\beta$ -amylase (200,000). Dextran blue (2,000,000) and vitamin B<sub>12</sub> (1,355.4) were used to determine the void volume ( $V_o$ ) and total volume ( $V_t$ ), respectively. A calibration curve between log molecular weights of protein markers and the partition coefficient values,  $K_{av}$ , was constructed.



**Figure 4. Isoelectric point determination of GLOD by isoelectric focusing.** The standard pI markers were: A, amyloglucosidase (3.5); B, soybean trypsin inhibitor (4.55); C,  $\beta$ -lactoglobulin A (5.20); D, bovine carbonic anhydrase B (5.85); E, human carbonic anhydrase B (6.55); F, myoglobin-acidic band (6.85); G, myoglobin-basic band (7.35); H, lentil lectin-acidic band (8.15); I, lentil lectin-middle band (8.45); J, lentil lectin-base band (8.65); K, trypsinogen (9.30).



**Figure 5. Effect of pH on GLOD activity (a) and stability (b).**

(a) GLOD activity was assayed in various buffers with different pH values.

(b) (a) The enzyme was assayed in various buffers at different pH values by the method as described in the text.

(b) The enzyme was incubated in various buffers at the pH values indicated.

After incubation at 37°C for 30 min, the remaining activities were determined by the method described in the text. The buffers (0.1 M) used were: acetate buffer (pH 3.5-6.0) (l), potassium phosphate buffer (pH 6.0-7.5) (u), Tris-HCl buffer (pH 7.5-9.0) (p), and glycine-NaOH buffer (pH 9.0-10.0) (n).

### Isoelectric point estimation

The isoelectric point was determined by using PhastGel<sup>®</sup> IEF 3-9 (Amersham Biosciences, Uppsala, Sweden). The standard pI markers consisted of amyloglucosidase (3.5), soybean trypsin inhibitor (4.55),  $\beta$ -lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), myoglobin-acidic band (6.85), myoglobin-basic band (7.35), lentil lectin-acidic band (8.15), lentil lectin-middle band (8.45), lentil lectin-base band (8.65) and trypsinogen (9.30). Plots of the distances of the protein markers from the anode and their pI's were constructed and fitted using linear regression. The pI of the GLOD was estimated using a regression equation.

## RESULTS

### Enzyme production

The extracellular GLOD was produced in wheat bran precultivation medium (Böhmer et al. 1989). GLOD activity obtained was 13.79 mU/ml. The composition of the medium was modified to formulate the medium that promoted the higher GLOD production. The effect of MSG added in the medium to the GLOD productivity was also investigated. There was no improvement in the enzyme productivity by adding glucose as a carbon source. The optimal GLOD production medium containing 2.0% wheat bran, 0.5% NaCl and 0.5% MSG was selected and named wheat bran medium. The maximum GLOD production was obtained from the cultivation of *Streptomyces* sp. 18G in wheat bran medium at 30°C for 60 hrs with shaking at 200 rpm.

### Purification of GLOD

GLOD was purified from an extract of *Streptomyces* sp. 18G cultured on wheat bran medium. The procedure included precipitation with ammonium sulphate, column chromatography on SP-SepharoseFF, Q-SepharoseFF and a high resolution gel filtration on Superdex 200 HR 10/30 as described above. Table 1 summarizes the purification of the enzyme. The overall purification was 990 fold with a yield of 16.65%. The purified enzyme showed a single band in SDS-PAGE and had a specific activity of 152.35 U mg<sup>-1</sup> (Figure 1).

### Molecular weight and subunit structure

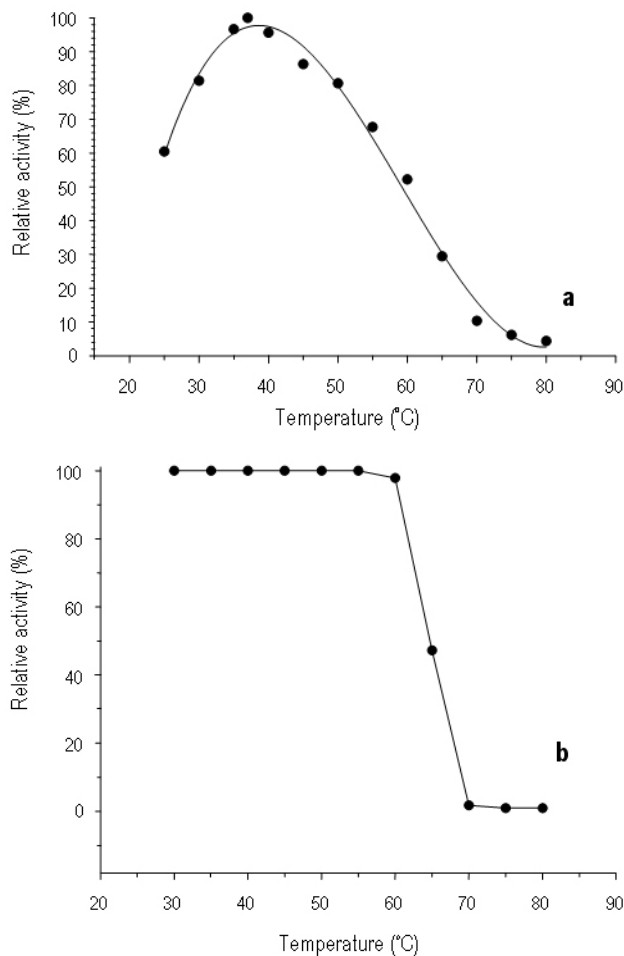
The relative molecular weight ( $M_r$ ) of the native enzyme was estimated to be approximately 120,000 by Superdex 200 HR 10/30 gel filtration chromatography (Figure 2). The subunit structure of the enzyme was analyzed by SDS-PAGE in 3 different polyacrylamide separating gel concentrations, i.e. 10%, 12% and 14% using a 4% stacking gel. Molecular weight of the enzyme subunits calculated from the three regression equations at different gel concentrations were 59,816, 63,252 and 60,044, respectively (Figure 3). The molecular weight of the GLOD subunit was estimated to be 61,000. Since the native enzyme was approximately twice the size of the enzyme subunit, the results suggested that the enzyme consisted of two identical subunits.

### Isoelectric point

The isoelectric point of the purified GLOD was estimated to be 8.5 by isoelectric focusing (Figure 4).

### Optimal pH and pH stability

Figure 5A shows the pH-activity profile of GLOD. The enzyme showed maximum activity in the pH range from 7.0 to 7.4. The enzyme was more stable in alkaline pH than in acidic pH (Figure 5B).



**Figure 6.** Effect of temperature on GLOD activity (A) and stability (B).

(a) The enzyme activity was assayed at various temperatures by the method as described in the text.

(b) The enzyme was incubated in 0.1 M potassium phosphate buffer pH 7.4 at various temperatures for 1 hr. The residual activity was investigated by the method described in the text.

### Optimal temperature and thermal stability

As illustrated in [Figure 6A](#), GLOD activity showed maximum activity at 37°C under standard assay conditions described above. Thermal stability of GLOD was determined by incubating the enzyme in 0.1 M potassium phosphate buffer pH 7.4 at various temperatures for 1 hr. The enzyme was relatively stable from 30 to 55°C. At 65°C, the enzyme showed approximately 50% of the original activity. The enzyme was completely inactivated at 75°C.

### Substrate specificity

The activity of GLOD on various amino acids was investigated. [Table 2](#) illustrates that L-glutamate was almost exclusively oxidized. In addition to L-glutamate, D-glutamate and L-aspartate were oxidized but with relative activities of 0.79% and 0.53%, respectively. The activities

on other amino acids tested were undetectable.

## DISCUSSION

An extracellular GLOD was isolated from *Streptomyces* sp. 18G. The enzyme was efficiently produced in wheat bran medium. The enzyme was purified approximately 990-fold from the culture broth with 16.65% yield. The specific activity was 152.36 U mg<sup>-1</sup>. The enzyme obtained in this study is different from those reported in previous studies in several aspects. The native enzyme has a molecular weight of approximately 120,000 Da with 2 identical subunits. The results were in accordance with previous studies by Böhmer et al. 1989 and Patel et al. 2000. However, Kusakabe et al. 1983 reported that GLOD from *Streptomyces* sp. X-119-6 had a molecular weight of approximately 140 kDa and consisted of 3 types of subunits, a, b and g with molecular weights of approximately 44, 19 and 9 kDa, respectively. Recently, another GLOD with three subunits was obtained from *S. platensis* NTU3304 (Chen et al. 2001). The pI of GLOD from *Streptomyces* sp. 18G was estimated to be 8.5 which differed from those obtained from *S. endus* and *Streptomyces* sp. X-119-6 with pI 6.2 in previous studies (Kusakabe et al. 1983; Böhmer et al. 1989). The pH-activity and temperature-activity profiles suggested that the optimal pH ranged from 7.0 to 7.4 and the optimal temperature was 37°C, which is applicable for glutamate determination under physiological conditions. In addition, the enzyme shows almost exclusively oxidize L-glutamate with lower extent on other amino acids tested. However, a low GLOD production was obtained from *Streptomyces* sp. 18G in this study. This result implies that control of the culture conditions, *i.e.* pH, salts, etc. may facilitate GLOD production. Arima and colleagues (2003) were successful in cloning and expressing the gene encoded for the enzyme in *Escherichia coli*. The report showed that proteolysis of the enzyme by metalloendopeptidase from *Streptomyces griseus* (Sgmp) could stabilize the recombinant enzyme and improved its catalytic efficiency at various pH. The report suggests that the DNA recombination technology can improve the GLOD production and stability.

Based on the results of this study, the GLOD from *Streptomyces* sp. 18G may have a potential for development of analytical systems for the specific determination of L-glutamate such as biosensors or kits for clinical diagnosis, bioprocess monitoring and food quality control. Additional studies are needed to obtain deeper insight into catalytic and physiochemical properties of the enzyme. Besides, molecular biology and bioprocess control may help promoting the production and stabilization of the enzyme.

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