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Identification of Catalytically Essential Amino Acid Residues and Immobilization of Glutamate Dehydrogenase from *Rumex* Cotyledons

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Abstract: The present study was designed to characterize and immobilize the glutamate dehydrogenase (GDH, EC 1.4.1.2) from *Rumex dentatus* cotyledons. The enzyme from *Rumex dentatus* was purified with specific activity of 145 U mg^{-1} protein. The indispensable role of arginine, lysine and tyrosine at the active site of the enzyme was demonstrated through chemical modification by 1,2-cyclohexanedione (CHD), trinitrobenzenesulfonic acid (TNBS) and tetranitromethane (TNM), respectively. The three modifiers were inactivated GDH enzyme with pseudo-first order kinetics and second order-rate constants of 22, 0.70 and 0.5 mM^{-1} min^{-1}, respectively. Both α-ketoglutarate and NADH offered GDH a protection against the inactivation. These studies suggested the involvement of arginine, lysine and tyrosine residues in the enzyme catalysis. GDH immobilized on gelatin beads via cross-linking with glutaraldehyde. The resulting immobilized enzyme was stored at 4°C for 10 days without loosing its activity. K_m of immobilized enzyme increased while V_{max} reduced compared to the free one. The immobilization of GDH resulted in a shift of pH optimum from 7.5 to 8.0. The optimum temperatures of the free and immobilized enzyme were 45°C and 60°C, respectively. The immobilized enzyme has a long life as compared with the native enzyme.

Key words: Glutamate dehydrogenase, *Rumex dentatus*, purification, immobilization, characterization, active groups

INTRODUCTION

GDH (EC. 1.4.1.2.) has been found in all organisms examined. This enzyme is highly conserved from bacteria to man. GDH has been investigated in microorganisms (Koike *et al*., 1996; Katranushkova *et al*., 1997; Camardella *et al*., 2002; Bhuiya *et al*., 2005; Williams *et al*., 2006); higher plants (Osuji and Madu, 1997; Turano *et al*., 1996; Frechilla *et al*., 2002) and human (Fang *et al*., 2002).

The enzyme of higher plants is abundant in storage organs of many crop species (Lea, 1997) GDH catalyzes the reversible amination of α-ketoglutarate to glutamate. The pathway of ammonia assimilation in higher plants is well established via the operation of the two enzymes glutamine synthetase (GS, EC. 6.3.1.2) and glutamate synthase (GOGAT, E C.1.4.1.13) (Taiz and Zeiger, 1998; Paradisi *et al*., 2005).

The role of GDH in higher plants has been the subject of continuous debate. There is now a consensus of opinion that the enzyme may be important in the deamination of glutamate to yield ammonia and 2-oxoglutarate, particularly during times of stress or senescence, although a role in ammonia assimilation is still proposed (Osuji and Madu, 1997). It is reported that the GDH enzyme is involved in the oxidative deamination process to provide carbon skeletons to the citric acid cycle

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(Lea, 1997; Heeschen *et al*., 1977). The demonstration that NADH-GDH is more efficient than GOGAT in the synthesis of L-glutamate in maize (Osuji and Madu, 1995) suggests that the enzyme may be involved in the salvage of the NH_4^+ during phytochemical defense response.

GDH is located in the mitochondria (Turano *et al*., 1996; Fang *et al*., 2002). The occurrence of GDH activity in the cytosol in addition to mitochondria reported for several plants (Heeschen *et al*., 1997), however it was suggested that the cytosolic activity was due to broken mitochondria. The enzyme is multi-isoenzyme in many plant tissues (Loulakakis and Roubelakis-Angelakis, 1991). Thus, GDH purified and characterized in microorganisms and higher plants. However, little information is available concerning the essential amino acids for the catalytic activity of GDH from *Rumex* cotyledons. Thus, the present work describes kinetic and chemical modification of GDH. In addition, the present study aimed to evaluate the characteristics of the free and immobilized enzyme from *Rumex* as C_3-plants.

MATERIALS AND METHODS

Seed Germination

Seeds of *Rumex dentatus* were surface sterilized with 0.1% NaOCl for about 2 min, washed thoroughly with distilled water and soaked in distilled water overnight. Seeds were sown in 20×15 cm plastic seed trays with an unperforated base, lined with a double layer of Whatman No. 1 filter paper, moistened with distilled water. The trays were incubated at 25°C with irradiance of 8 μmol m^{-2} sec^{-1} for 5 days.

Enzyme Extraction

Cotyledons (60 g) of 5 days seedlings were extracted in a medium containing 100 mM phosphate buffer (pH 7.5), 1 mM EDTA, 1 mM DTT and 0.5% Triton X-100 (v/v) at 4°C. The homogenate was filtered through four layers of cheesecloth. The resulting clear filtrate was used as the crude enzyme preparation.

Enzyme Purification

The purification procedure of GDH was carried out according to the method of El-Shora and Abo-Kassem (2001).

Enzyme Assay

GDH activity was determined spectrophotometrically at 340 nm. The reaction mixture contained in a volume of 3 mL, 100 mM Tris-HCl (pH 7.5), 5 mM α-ketoglutarate, 20 mM NH_4Cl, 1 mM $CaCl_2$ and 2 mM NADH. The reaction was started by addition of the enzyme. One unit (U) of enzyme activity is defined as the amount of enzyme required to convert 1 μmol NADH to NAD min^{-1}.

SDS/Polyacrylamide-Gel Electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed by the procedure of Laemmli (1970). Proteins were stained with Coomassie Brilliant Blue. The molecular mass of the native enzyme was estimated by molecular sieve chromatography on a Sephacryl S-200 column calibrated with ferritin (450 kilodaltons [kDa]), catalase (220 kDa), aldolase (158 kDa), bovine serum albumin (68 kDa) and ovalbumin (45 kDa).

Protein Determination

Protein was determined by the method of Lowry *et al*. (1951) after precipitation with equal volumes of 20% TCA and using bovine serum albumin as standard.

Chemical Modification of GDH

Chemical modification of GDH by CHD was carried out according to the method of Adak *et al*. (1996). Modification by TNBS was performed by the method adopted by Hartleib and Ruterjans (2001). Modification by TNM was carried out according to the method adopted by El-Shora (2001a).

Preparation of Immobilized GDH

The GDH was immobilized according to the method of El-Shora (2001b). Gelatin powder (5-10% w/v) used for the immobilization of urease enzyme was swelled in 10 mL (50 mM L^{-1}) phosphate buffer (pH 7.0) and heated at 50°C for 5 min for complete solubilization of gelatin. The mixture was cooled and enzyme (0.8 mg protein in 0.2 mL of buffer) was added. After through mixing of the enzyme, required amount (0.6% w/v) of organic cross- linker, gluteraldehyde was added. The mixture was stirred constantly at 28°C and poured on a (7×4) cm^2 glass plate to prepare a thin film of the enzyme. The film was stored at 4°C for 18 h for complete cross-linking. The immobilized enzyme film was washed thoroughly with 50 mM L^{-1} phosphate buffer (pH 7.0) and cut into small blocks before subsequent experiments.

RESULTS

GDH was purified from *Rumex* cotyledons by a process including ammonium sulphate (55-75%), DEAE-cellulose, CM-Sephadex and Sephacryl S-100 (Table 1). The purified enzyme was judged to be homogeneous (Fig. 1) on the basis of SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The detectable activity was associated with a single protein band observed in the stained SDS-PAGE. All the following experiments were carried out with the purified enzyme.

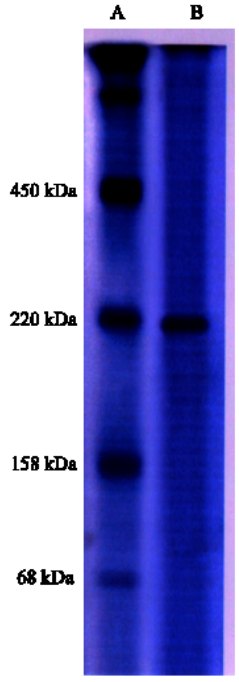

Fig. 1: Gel electrophoresis of glutamate dehydrogenase from marrow cotyledons. A: Standards markers and B: The pure enzyme from Sephacryl S-200 column

Table 1: Summary of the purification procedure of GDH from *Rumex* cotyledons. The enzyme was extracted from cotyledons of 5-day old seedlings

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Recovery (%)	Purification factor
Crude extract	295.3	185.3	0.6	100.0	1.0
$(NH_4)_2SO_4$(55-75%)	57.1	103.3	1.8	55.7	3.0
DEAE-Cellulose	5.8	57.3	9.9	30.9	16.5
CM-Sephadex	0.5	27.9	55.8	15.1	93.0
Sephacryl S-200	0.1	14.5	145.0	7.8	241.7

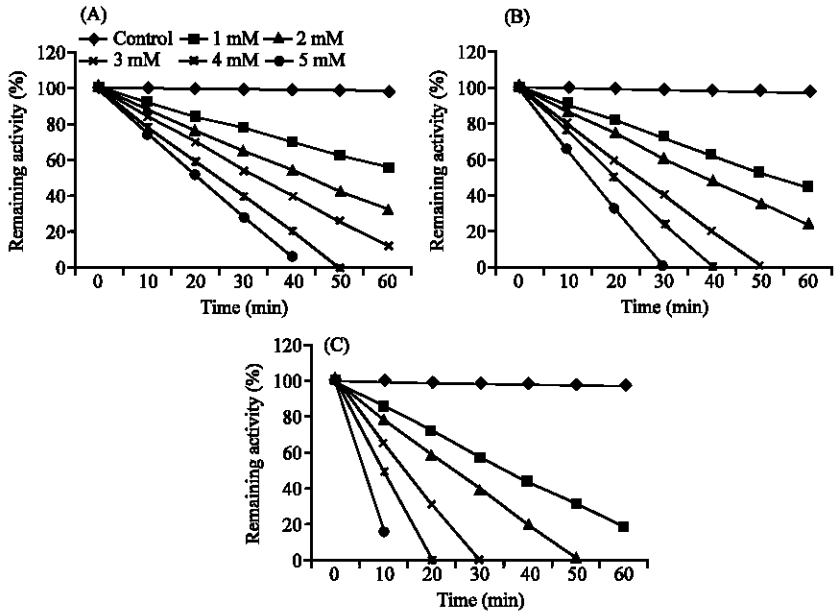

Fig. 2: Kinetics of the inactivation of GDH by: (A) CHD, (B) TNBS and (C) TNM

The time course of inactivation of GDH by various concentrations of CHD is shown in Fig. 2A. Inactivation of GDH by CHD followed pseudo-first-order kinetics and displayed second -order rate constant of 22 mM^{-1} min^{-1} (Fig. 3A).

Treatment of GDH with TNBS also resulted in time-dependent loss of enzymatic activity. The inactivation followed pseudo-first-order kinetics (Fig. 2B). Kinetic analysis of inactivation yielded a second-order rate constant of 0.7 mM^{-1} min^{-1} (Fig. 3B).

The time course of inactivation of GDH by various concentrations of TNM is shown in Fig. 2C. The inactivation follows pseudo-first-order kinetics and kinetic analysis of inactivation yielded a second-order rate constant of 0.5 mM^{-1} min^{-1} (Fig. 3C).

Incubation of GDH with 5 mM of α-Ketoglutarate or NADH before adding CHD, TNBS or TNM protected the enzyme against their effect (Table 2). α-ketoglutarate was the better protector in the three cases.

The optimum pH value was 7.5 for the free GDH. Immobilization of GDH resulted in a shift of pH optima from 7.5 to 8.0. Also, pH curve of immobilized enzyme was found to be broader in comparison with that of the free GDH as shown in Fig. 4.

Both the free and immobilized GDH were kept in different pH buffer solutions (pH 3-10) for 60 min (Fig. 5). The results show that the immobilized GDH was stable from pH 5 to 9, while native enzyme was stable from pH 6 to 8.

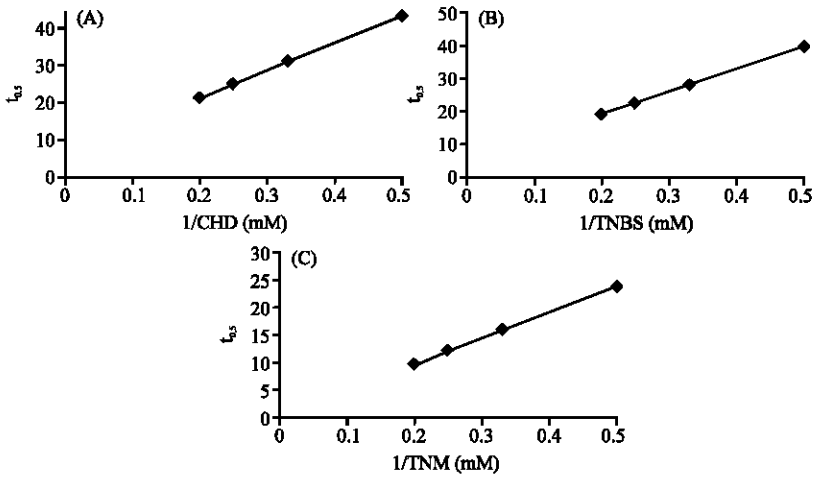

Fig. 3: Relation between $t_{0.5}$ and reciprocal of inhibitor concentration. (A) CHD, (B) TNBS and (C) TNM

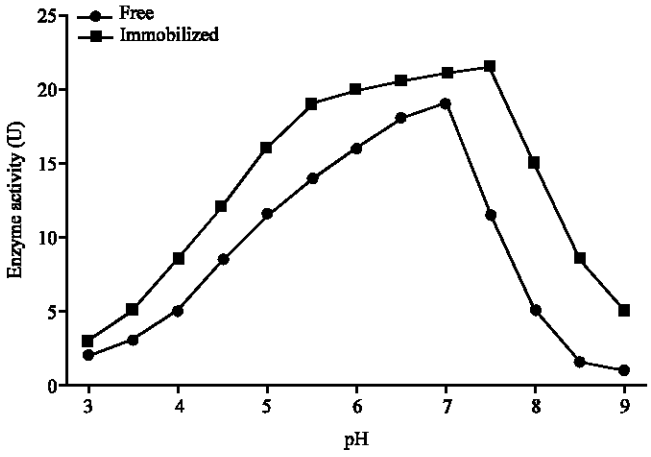

Fig. 4: Effect of pH on free and immobilized GDH from *Rumex* cotyledons

Table 2: Protection of GDH by 5 mM of α-ketoglutarate or NADH against inactivation by CHD, TNBS and TNM

Treatment	Activity (U)	Activity (% control)
Control	18.6±0.8	100.0
E+CHD	5.2±0.5	28.2
E+α-ketoglutarate+CHD	16.5±0.7	88.5
E+NADH+CHD	11.7±0.3	62.7
E+TNBS	3.9±0.8	21.1
E+α-ketoglutarate+TNBS	14.6±0.6	78.4
E+NADH+TNBS	11.6±0.9	62.6
E+TNM	2.8±0.7	15.3
E+α-ketoglutarate+TNM	13.5±0.2	72.3
E+NADH+TNM	9.3±0.5	50.1

Free GDH showed an optimal temperature at 40°C whereas its immobilized counterpart demonstrated the optimal activity at 60°C (Fig. 6). Comparison of thermostability also revealed an advantage of immobilized GDH. Little difference in the activity of free and immobilized GDH was

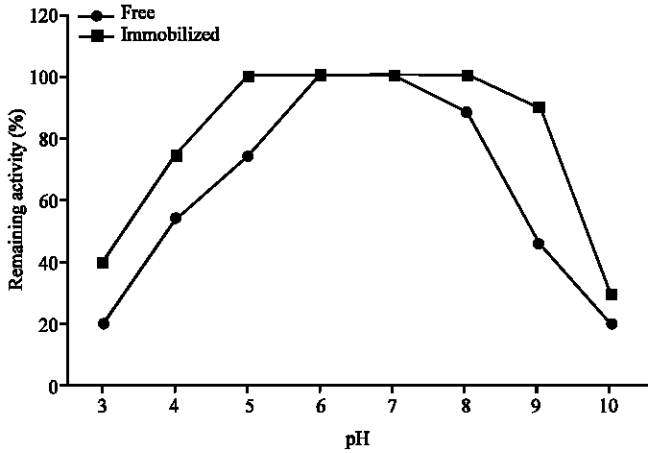

Fig. 5: Effect of pH on the stability of the free and immobilized GDH from *Rumex* cotyledons

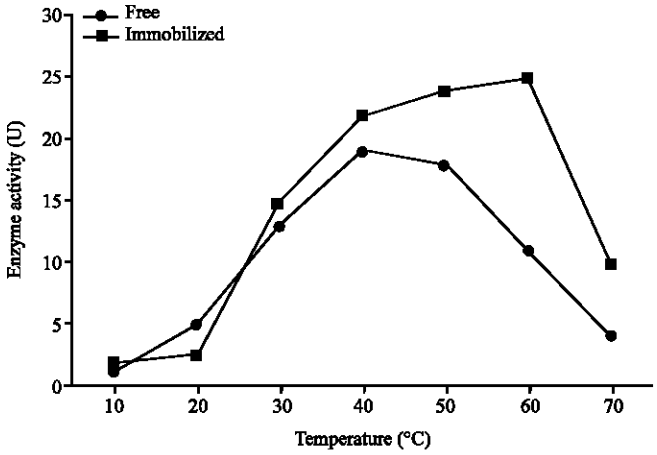

Fig. 6: Effect of temperature on the free and immobilized GDH from *Rumex* cotyledons

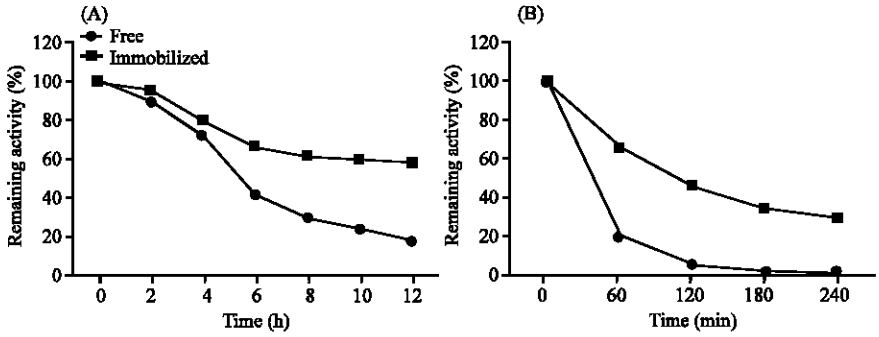

Fig. 7: Temperature stability of free and immobilized GDH from *Rumex* cotyledons. (A) at 50°C and (B) at 60°C

found for 4 h of incubation at 50°C. However, after 12 h of incubation at 50°C, free GDH had lost 80% of its initial activity, whereas immobilized GDH had lost only 40% (Fig. 7a). An increase in

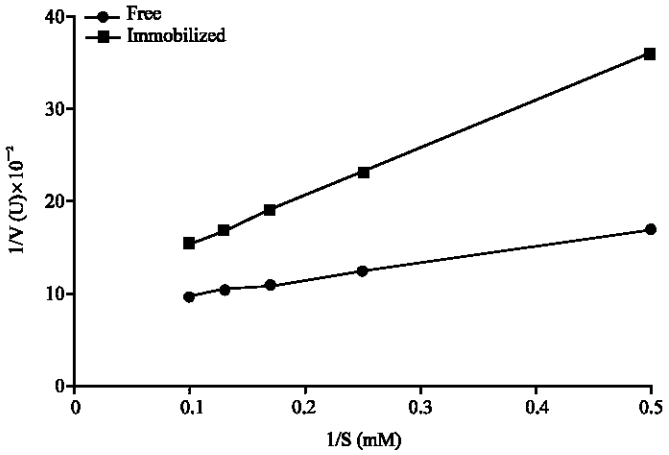

Fig. 8: Lineweaver-Burk plot for GDH from *Rumex* cotyledons

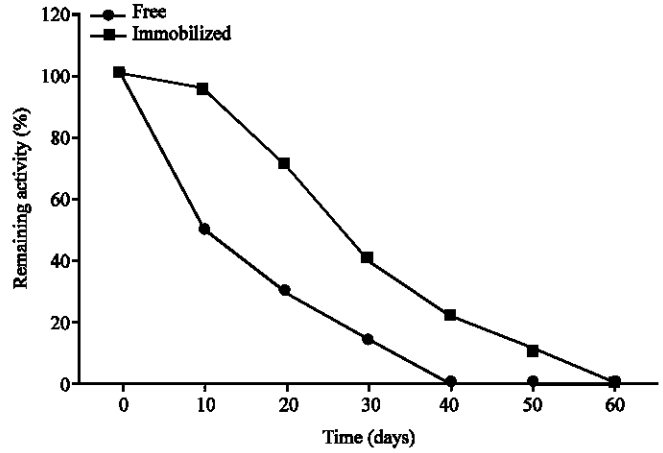

Fig. 9: Long-term stability at 4°C of the free and immobilized GDH from *Rumex* cotyledons

temperature to 60°C was most destructive, but again, after 2 h of incubation the immobilized enzyme retained more than 42% of initial activity whereas only trace activity of the free enzyme could be detected (Fig. 7b).

GDH showed classical Michaelis-Menten kinetics with respect to α-ketoglutarate (Fig. 8). The apparent K_m values for α-ketoglutarate were 3.3 mM and 7.7 mM for the free and immobilized enzymes. Also, V_{max} values were 14 and 10 μmol min^{-1} for the free and immobilized enzymes.

Long-term stability of the free and immobilized GDH was investigated. Both enzymes were kept for 60 days at 4°C and the enzyme activity was measures every 10 days. The activity of the free GDH started to decrease continuously with time and on day 40 no activity was detected. However, the activity of immobilized GDH was almost the same as the initial activities up to 10 days. After 10 days the enzyme activity started to decrease with time and almost no activity was observed on day 60 (Fig. 9).

DISCUSSION

GDH was purified from *Rumex* cotyledons with 241.7-fold purification and specific activity of 145 U mg^{-1} protein. This value is higher than that obtained by other investigators (Koike *et al*., 1996;

El-Shora, 1993; El-Shora and Abo-Kassem, 2001). The detectable activity was associated with a single protein band observed in stain gels and by SDS-PAGA, a subunit molecular weight of 52 000 kDa was determined which is in harmony with that reported for the enzyme from *Sphagnum* and *Salinibacter ruber* (Heeschen *et al*., 1997; Bonete *et al*., 2003).

One of the principal goals of the present study is the elucidation of functional groups that are responsible for the enzyme catalysis. The present results show that arginine-specific modifier CHD, lysine-modifier TNBS and tyrosine-modifier TNM inactivated the GDH in a time and concentration dependent manner causing an exponential decline in activity. This inactivation of GDH by three tested reagents indicates the essentiality of three residues for the enzyme catalysis, which is in agreement with the results for glutamate dehydrogenase from other sources as reported by other investigators (Perez-Pomares *et al*., 1999; Ahn *et al*., 2000).

Protection of GDH by α-ketoglutarate or NADH supports the finding that arginine, lysine and tyrosine are essential for GDH catalysis. The optimum pH of free GDH is 7.5, which is in harmony with the results of El-Shora and Abo-Kassem (2001). Also, the immobilized enzyme was stable form pH 5 to 9 compared to the stability of the free GDH at pH 6 to 7.5. On immobilization, the optimum pH shifted to 8.0. Broadening of the pH curve, shifts of pH-optimum and enhanced tolerance to unfavorable conditions are well known properties of many immobilized enzymes (Davis and Burns, 1992; Rogaiski *et al*., 1999).

The fixed position of immobilized enzyme on the surface of the carrier thought to prevent extensive distortion of the native conformation of the enzyme protein caused by extreme pH (Davis and Burns, 1992; Pye and Chance, 1976) The optimum temperature of the immobilized GDH was increased to 60°C, which is higher than 45°C recorded with the free GDH. The thermostability of immobilized GDH could be due to diminished autolysis of the enzyme fixed to the support (Kamakura and Kaetsu, 1984).

The increase in immobilized enzyme K_m can be mainly attributed to conformational changes, variations in the microenvironment and, in the case of inclusion in gel, diffusion effects of the substrate and/or products (Chibata *et al*., 1987). The reduction in V_{max} for immobilized enzymes as compared to the free GDH is usual. This is mainly due to their partial inactivation caused by less favorable conditions of catalysis following the immobilization process (Palmer, 1985).

The immobilized GDH expressed long-term stability at 4°C compared to the free enzyme. This could be related to the rigidity of the conformation of the enzyme molecules resulting from binding to the matrix (Huckel *et al*., 1996).

In conclusion, GDH was purified and successfully immobilized on gelatin matrix. The immobilized GDH was more active in strongly acidic and alkaline pH, more stable and active at high temperature. These properties, as well as the shift of optimal pH towards alkaline values and the increase in the optimal reaction temperature to at least 60°C are good reasons to apply such systems to practical purposes. The present paper offers an interesting field for further research, especially structural and conformational problems of GDH.

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