

## Purification and some catalytic properties of glucose-6-phosphate dehydrogenase isoforms from barley leaves

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### Abstract

The cytosolic and chloroplastic isoforms of glucose-6-phosphate dehydrogenase (G<sub>6</sub>PDH) were separated and purified from barley leaves (*Hordeum vulgare* L.). In etiolated leaves, only the cytosolic isoform was expressed. The molecular mass of the cytosolic enzyme, G<sub>6</sub>PDH<sub>1</sub>, was 112±8 kDa and that of the chloroplast enzyme, G<sub>6</sub>PDH<sub>2</sub>, was 136±7 kDa. The K<sub>m</sub> values for glucose-6-phosphate and NADP were 0.133 and 0.041 mM for G<sub>6</sub>PDH<sub>1</sub>, and 0.275 and 0.062 mM for G<sub>6</sub>PDH<sub>2</sub>, respectively. The pH optimum was 8.2 for G<sub>6</sub>PDH<sub>1</sub> and 7.8 for G<sub>6</sub>PDH<sub>2</sub>. The enzyme is absolutely specific for NADP. NADPH is a competitive inhibitor of the G<sub>6</sub>PDH<sub>1</sub> in respect to glucose-6-phosphate (G<sub>6</sub>P) and NADP (K<sub>i</sub> = 0.050 and 0.025 mM, respectively). NADPH is a competitive inhibitor of the G<sub>6</sub>PDH<sub>2</sub> in respect to NADP (K<sub>i</sub> = 0.010 mM), but a non-competitive inhibitor in respect to the G<sub>6</sub>P. ADP, AMP, UTP, NAD, and NADH had no effect on the activity of G<sub>6</sub>PDH. ATP inhibited the G<sub>6</sub>PDH<sub>2</sub> activity.

*Additional key words:* competitive and non-competitive inhibition; cytosolic and chloroplastic forms; etiolated leaves; *Hordeum vulgare*; K<sub>i</sub>; K<sub>m</sub>; NADP.

### Introduction

G<sub>6</sub>PDH (EC 1.1.1.49) catalyses the conversion of G<sub>6</sub>P to 6-phosphoglucono-δ-lactone. G<sub>6</sub>PDH is a key limiting enzyme in the pentose phosphate pathway (Williams 1980, Copeland and Turner 1987). In several C<sub>3</sub>-plants, many enzyme activities of sugar phosphate metabolism have been demonstrated in both the chloroplasts and the cytosol. All such enzymes of green leaf cells could be separated into two isoforms, one located in the chloroplasts and the other in the cytosol (Schnarrenberger *et al.* 1992, 1995). The plastid G<sub>6</sub>PDH isoenzyme seems to resemble the cytoplasmic form in several general properties, including molecular mass, kinetic properties, and control of certain effectors in green leaves such as spinach (Lendzian 1980, Schnarrenberger *et al.* 1995) and in non-green plant tissue such as castor bean endosperm (Miernyk 1992). Although the plant enzyme is less studied, the available data indicate different regulatory

mechanisms as compared to animal tissues and micro-organisms. In particular, the chloroplast G<sub>6</sub>PDH is regulated by irradiation through redox reactions of thiol groups—inversely to the other irradiation-regulated enzymes of the Calvin cycle, but the cytosol G<sub>6</sub>PDH is not (Scheibe and Anderson 1981, Graeve *et al.* 1994).

However, comparative characterisation of cytosolic and plastidic isoforms of G<sub>6</sub>PDH from plant tissues is very rare. Nevertheless, elucidation of peculiarities of different isoform function may explain some aspects of saccharide metabolism in cytosol and chloroplast.

Hence, in the present paper we describe the separation of two isoforms of G<sub>6</sub>PDH from barley leaves and their purification to homogenous state. That provides comparison of catalytic properties and possibilities of activity regulation of cytosolic and plastidic G<sub>6</sub>PDH isolated from leaf tissue of the same monocotyledonous plant.

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Abbreviations: G<sub>6</sub>P – glucose-6-phosphate; G<sub>6</sub>PDH – glucose-6-phosphate dehydrogenase.

## Materials and methods

The leaves of 10-d-old barley leaves (*Hordeum vulgare* L.) grown in a greenhouse under hydroponic conditions and daylight irradiation at photon flux density of  $0.15 \text{ J s}^{-1} \text{ m}^{-2}$  and at  $25^\circ \text{C}$  were used.

The activity of  $G_6PDH$  was measured spectrophotometrically using the spectrophotometer *SP-46* (Lomo, St. Petersburg, Russia) at 340 nm by the rate of NADP reduction during the conversion of  $G_6P$  to 6-phosphogluconolactone. The reaction mixture consisted of 0.05 M Tris-HCl, pH 7.5, 2 mM  $G_6P$ , 0.25 mM NADP, 0.5 mM Na-EDTA, and 7.5 mM  $MgCl_2$ . Enzyme reaction was started by adding glucose-6-phosphate into the cuvette. Protein concentration was determined according to Lowry *et al.* (1951).

Electrophoresis was conducted in 7.5 % polyacrylamide gels under non-denaturing conditions according to Davis (1964). Proteins were stained with Coomassie Brilliant Blue R-250 (Maurer 1968). Gel staining for enzyme detection by activity was performed according to Gabriel (1971). The molecular mass of the enzymes was estimated by chromatography on *Toyopearl HW-55* (Toyo-Soda, Tokyo, Japan). The calibration curves were constructed using the following markers: ferritin from horse spleen (450 kDa), aldolase (160 kDa) and phosphoglucomutase (66 kDa) from rabbit muscles, ovalbumin (45 kDa), myoglobin (17.8 kDa), and cytochrome *c* (12.5 kDa).

Original methods were used for purification of cytosolic and chloroplastic isoforms. Purification of  $G_6PDH_1$  was performed as follows. Step 1: Leaves (15 g) were homogenised using a chilled mortar and pestle in two volumes of extraction buffer containing 0.04 M Tris-HCl, pH 7.5, 1 mM Na-EDTA, 0.05 M 2-mercaptoethanol, 24 mM NADP, 2 kg  $\text{m}^{-3}$  polyvinylpyrrolidone, and 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at  $9\,000\times g$  for 15 min. Step 2: The enzyme preparation was fractionated by ammonium sulphate precipitation, using the cut between 30 and 60 % saturation at pH 7.5. After centrifugation at  $15\,000\times g$  for 30 min, the precipitate was re-suspended in a minimum volume of the extraction buffer. Step 3: The protein preparation was

purified from low-molecular-mass contaminants by gel-filtration on a *Sephadex G-25* (fine) column ( $1.4\times 30 \text{ cm}$ ). The column was equilibrated and the enzyme was eluted with 10 mM Tris-HCl, pH 7.5, containing 1 mM Na-EDTA and 2 mM dithiothreitol. Step 4: The enzyme preparation was applied to a DEAE-cellulose column ( $1.1\times 10.0 \text{ cm}$ ) equilibrated with the same column buffer as before. Proteins were eluted with  $110 \text{ cm}^3$  of 0 to 0.2 M KCl gradient in column buffer. Fractions of  $2\text{--}3 \text{ cm}^3$  were collected. Step 5: The enzyme preparation was then applied to a *Sephadex G-150* column ( $1.5\times 75.0 \text{ cm}$ ) equilibrated with the same column buffer as before. Proteins were eluted using a peristaltic pump (Pharmacia, LKB – Pump 1) at a flow rate of  $8.33 \text{ mm}^3 \text{ s}^{-1}$ .

Purification of the  $G_6PDH_2$  was the same as for  $G_6PDH_1$  during the first three steps. Subsequently, the enzyme preparation was applied to a DEAE-cellulose column ( $1.1\times 10.0 \text{ cm}$ ) equilibrated with column buffer as described above. Proteins were eluted with  $110 \text{ cm}^3$  of 0 to 0.2 M KCl gradient in column buffer and fractions of  $2\text{--}3 \text{ cm}^3$  were collected. When the first peak of  $G_6PDH$  activity was eluted, 10 mM fructose-1,6-bisphosphate was applied into the column buffer, and as a result, the second peak of  $G_6PDH$  activity was obtained. The resulting enzyme preparation was applied to *Sephadex G-150* column as described above. All procedures were performed in a cold chamber at 0 to  $4^\circ \text{C}$ .

The statistical processing of values was accomplished by standard procedures (Lloyd and Lederman 1984). Programs of linear and parabolic approximations (*Microsoft Excel 97*, *Harvard Graphics*) were used for graphical representations.

*Sephadex G-25* and *G-150* were obtained from Pharmacia (Uppsala, Sweden). DEAE-32-cellulose was from Whatman (Maidstone, United Kingdom). Tris and polyvinylpyrrolidone were purchased from Serva (Heidelberg, Germany). NADP,  $G_6P$ , and galactose-6-phosphate were obtained from Sigma (St. Louis, USA). Phenylmethylsulfonyl fluoride and dithiothreitol were from Merck (Darmstadt, Germany); other reagents were obtained from Russian manufacturers.

## Results and discussion

Separation of the two  $G_6PDH$ s was achieved by chromatography on DEAE-cellulose. Profiles of the  $G_6PDH$  activity and total protein are presented in Fig. 1.  $G_6PDH_1$ , the cytosol isoenzyme, was eluted with 0 to 0.2 M KCl gradient, and  $G_6PDH_2$ , the chloroplast isoenzyme, with 0.2 M KCl and 10 mM fructose-1,6-bisphosphate. The resulting enzyme preparations were used for further purification (Table 1). The procedure resulted in a 105-fold purification of the  $G_6PDH_1$  with specific activity of  $38.00$

$\text{mkat kg}^{-1}(\text{protein})$ . Native PAGE showed that, after gel-chromatography on *Sephadex G-150*, the fractions with maximum activity were homogeneous (Fig. 2). After staining with Coomassie Brilliant Blue, one band with an electrophoretic mobility  $R_f \sim 0.10$  was revealed in the gel. On the other hand, the procedure resulted in a 63-fold purification of the  $G_6PDH_2$  with specific activity of  $22.52 \text{ mkat kg}^{-1}(\text{protein})$ . PAGE after gel-chromatography on *Sephadex G-150* revealed a homogeneous protein band in

fractions with maximum enzymatic activity (Fig. 2). Its electrophoretic mobility was  $R_f \sim 0.24$ . Minor components were not detected. The degree of purification and the yield of both enzymes were low, due to extreme instability of the enzymes, especially of the chloroplast form.

Detection of isoenzymes by enzyme activity in native gels revealed that etiolated barley leaves contained a sin-

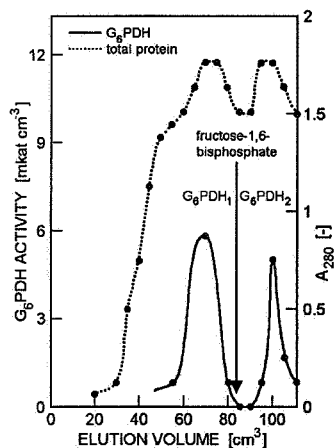


Fig. 1. Chromatography of the enzyme preparation from barley leaves on DEAE-cellulose. Profiles of the total protein and glucose-6-phosphate dehydrogenase ( $G_6PDH$ ) activity during chromatography are presented.

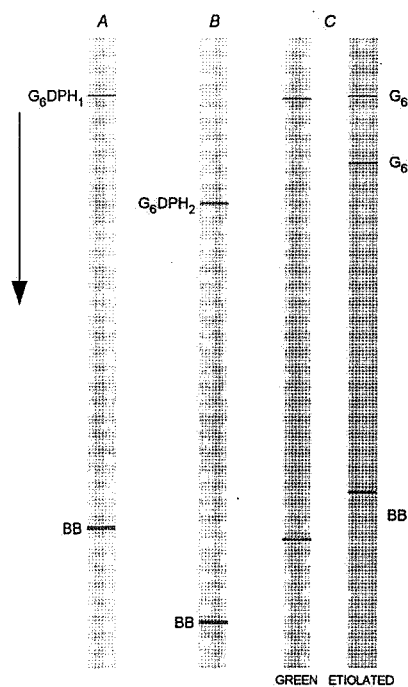


Fig. 2. Electrophoretic separation of (A) glucose-6-phosphate dehydrogenase 1 ( $G_6PDH_1$ ) and (B) glucose-6-phosphate dehydrogenase 2 ( $G_6PDH_2$ ) from barley leaves. C: the isoforms in green and etiolated leaves. BB - marker dye bromophenol blue. Arrow shows the direction of protein migration.

gle band ( $R_f \approx 0.1$ ) of  $G_6PDH$  activity, the mobility of which was similar to the  $R_f$  obtained for the  $G_6PDH_1$ . With green leaves two bands with  $R_f \approx 0.10$  and  $0.24$ , respectively, were obtained. The latter value was similar to the  $R_f$  of  $G_6PDH_2$ . Therefore  $G_6PDH_1$ , the cytosolic isoform, may be constitutively expressed and  $G_6PDH_2$ , the chloroplast isoform, is expressed only after irradiation (Fig. 2). Similarly, Schaewen *et al.* (1995) found that expression of the plastid enzyme takes place in potato leaves, but not in tuber tissue.

The isolation of purified proteins allowed study of physicochemical properties and regulatory properties of the  $G_6PDH$ s from barley leaves. The molecular mass of

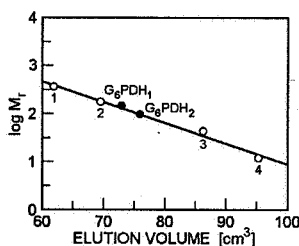


Fig. 3. Determination of molecular mass of glucose-6-phosphate dehydrogenase isoforms  $G_6PDH_1$  and  $G_6PDH_2$  from barley leaves by chromatography on *Toyopearl HW-65*. Marker proteins: ferritin from horse spleen (450 kDa) (1), aldolase from rabbit muscles (160 kDa) (2), ovalbumin (45 kDa) (3), and cytochrome *c* (12.5 kDa) (4).

the enzymes determined by gel-chromatography on *Toyopearl HW-65* was  $112 \pm 8$  for  $G_6PDH_1$  and  $136 \pm 7$  kDa for  $G_6PDH_2$  (Fig. 3). The molecular masses of microbial  $G_6PDH$  range between 100 and 120 kDa and of mammalian enzymes between 120 and 130 kDa (Levy 1979).

The pH optimum for the reaction catalysed by the  $G_6PDH_1$  was 8.2. The pK of ionogenic groups estimated

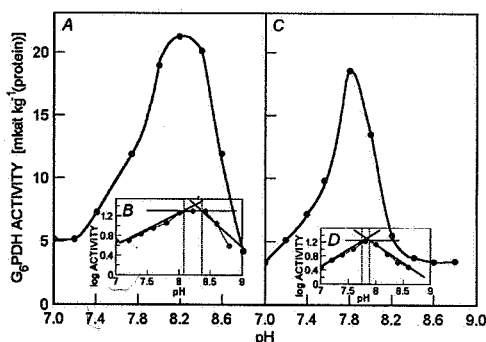


Fig. 4. pH dependence of glucose-6-phosphate dehydrogenase isoforms activity (A, C) and determination of pK values of functional groups from  $\{\log V: pH\}$  plot (B, D) for glucose-6-phosphate dehydrogenase 1,  $G_6PDH_1$  (A, B) and glucose-6-phosphate dehydrogenase 2,  $G_6PDH_2$  (C, D) (0.05 M Tris-HCl, pH 7.5, containing 0.5 mM Na-EDTA, 7.5 mM  $MgCl_2$ , 0.25 mM NADP, and 2 mM glucose-6-phosphate).

from dependence of log activity *versus* pH were 8.05 and 8.45. This is close to the pK of cysteine sulfhydryl groups. The G<sub>6</sub>PDH<sub>2</sub> has pH optimum of 7.8. Values of pK for ionogenic groups calculated from the (log activity; pH) dependence were 7.75 and 7.95. The first is close to the pK of imidazole group of histidine whereas the second is close to the cysteine sulfhydryl group (Fig. 4).

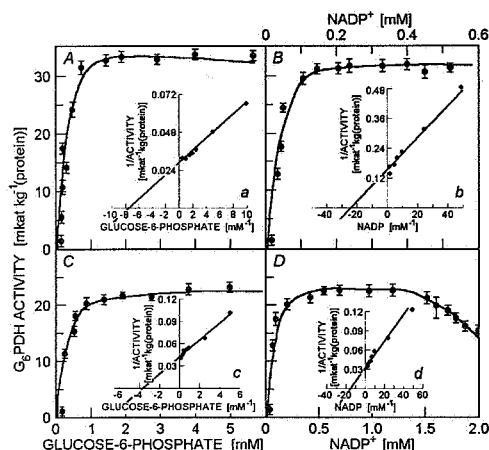


Fig. 5. Dependence of the reaction rate catalysed by glucose-6-phosphate dehydrogenase 1 (G<sub>6</sub>PDH<sub>1</sub>) (A, B) and glucose-6-phosphate dehydrogenase 2 (G<sub>6</sub>PDH<sub>2</sub>) (C, D) from barley leaves on glucose-6-phosphate concentration (A, C) (0.05 M Tris-HCl, pH 7.5, containing 0.5 mM Na-EDTA, 7.5 mM MgCl<sub>2</sub>, and 0.25 mM NADP) and NADP concentration (B, D) (0.05 M Tris-HCl, pH 7.5, containing 0.5 mM Na-EDTA, 7.5 mM MgCl<sub>2</sub>, and 3 mM glucose-6-phosphate). K<sub>m</sub> determination by the Lineweaver-Burk method for glucose-6-phosphate (a, c) and NADP (b, d).

The dependence of the G<sub>6</sub>PDH activity on substrate concentration followed the Michaelis-Menten kinetics. The K<sub>m</sub> values of G<sub>6</sub>PDH<sub>1</sub> and G<sub>6</sub>PDH<sub>2</sub> for G<sub>6</sub>P were 0.133 and 0.275 mM, respectively. The K<sub>m</sub> values of G<sub>6</sub>PDH<sub>1</sub> and G<sub>6</sub>PDH<sub>2</sub> for NADP were 0.041 and 0.062 mM, respectively (Fig. 5). A substrate inhibition of G<sub>6</sub>PDH<sub>2</sub>, however, occurred at high concentrations of NADP with a K<sub>i</sub> of 0.45 mM (Fig. 6). The reported K<sub>m</sub>

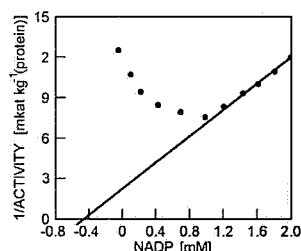


Fig. 6. Determination of the constant of NADP-caused inhibition of glucose-6-phosphate dehydrogenase 2 (G<sub>6</sub>PDH<sub>2</sub>) from barley leaves (0.05 M Tris-HCl, pH 7.5, containing 0.5 mM Na-EDTA, 7.5 mM MgCl<sub>2</sub>, and 3 mM glucose-6-phosphate).

values for NADP vary from 14 mM (black gram enzyme; Ashihara and Komamine 1976) to 330 mM (wheat germ enzyme; Mirfakhrai and Auleb 1989). The K<sub>m</sub> values for G<sub>6</sub>P vary from 9 mM (wheat germ enzyme; Mirfakhrai and Auleb 1989) to 18 mM (hazel cotyledons enzyme; Gosling and Ross 1979).

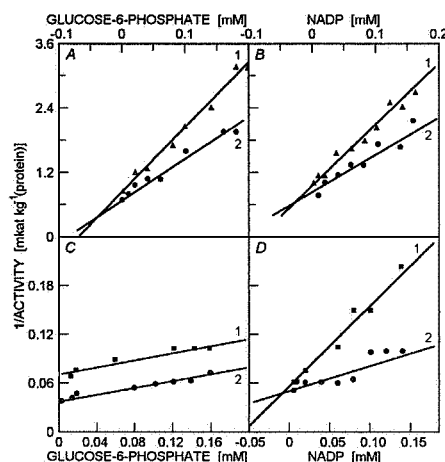


Fig. 7. Determination of the type and constants of NADPH-caused inhibition of glucose-6-phosphate dehydrogenase 1, G<sub>6</sub>PDH<sub>1</sub> (A, B) and glucose-6-phosphate dehydrogenase 2, G<sub>6</sub>PDH<sub>2</sub> (C, D) from barley leaves with respect to glucose-6-phosphate [0.05 M Tris-HCl, pH 7.5, containing 0.5 mM Na-EDTA, 7.5 mM MgCl<sub>2</sub>, 0.25 mM NADP, and the fixed concentrations of glucose-6-phosphate: 1 mM (1) and 2 mM (2)] (A, C) or to NADP [0.05 M Tris-HCl, pH 7.5, containing 0.5 mM Na-EDTA, 7.5 mM MgCl<sub>2</sub>, NADP, 2 mM glucose-6-phosphate, and the fixed concentrations of NADP: 0.10 mM (1) and 0.25 mM (2)] (B, D).

NAD had no appreciable effect on G<sub>6</sub>PDH isozymes. G<sub>6</sub>PDH was active only with NADP, being consistent with G<sub>6</sub>PDH of almost all other plants (Muto and Uritani 1972, Ashihara and Komamine 1976, Gosling and Ross 1979, Mirfakhrai and Auleb 1989). In this respect, plant G<sub>6</sub>PDH differs considerably from microbial enzymes, most of which are active both with NAD and NADP (Levy 1979). Sugar phosphates such as fructose-1,6-bisphosphate and glucose-1-phosphate also could not re-

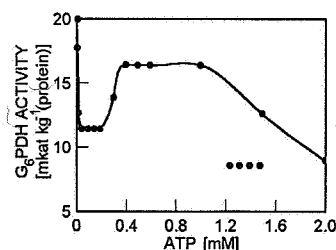


Fig. 8. Effect of ATP on the glucose-6-phosphate dehydrogenase 2, G<sub>6</sub>PDH<sub>2</sub> reaction rate (0.05 M Tris-HCl, pH 7.5, containing 0.5 mM Na-EDTA, 7.5 mM MgCl<sub>2</sub>, 0.25 mM NADP, and 2 mM glucose-6-phosphate).

place G<sub>6</sub>P in the standard assay system. NADPH is a competitive inhibitor of the G<sub>6</sub>PDH<sub>1</sub> with respect to G<sub>6</sub>P and NADP with  $K_i$  values of 0.050 and 0.025 mM, respectively. NADPH is a competitive inhibitor of the G<sub>6</sub>PDH<sub>2</sub> with respect to the NADP with a  $K_i$  of 0.010 mM, but in respect to the G<sub>6</sub>P it is a non-competitive inhibitor (Fig. 7). In comparison,  $K_i$  for NADPH with respect to NADP is 0.070 mM for the spinach stroma enzyme (Lendzian 1980) and 0.010 mM for the sweet potato enzyme (Muto and Uritani 1972). The concentration of NADPH in spinach chloroplast is about 0.5 mM (Takahama *et al.* 1981). Probably a considerable amount of the nucleotide is a protein bound *in vivo*. If this is so, then the control of activity of the enzyme by reduction charge (Lendzian and Bassham 1975) seems likely.  $K_i$  (NADPH) values reported for the enzyme from other sources range

from 0.7  $\mu$ M (*Saccharomyces carlsbergensis*) to 0.15 mM (rat adipose tissue) (Levy 1979).

ATP, ADP, AMP, UTP, NAD, and NADH at concentrations below 2 mM have no effect on the G<sub>6</sub>PDH<sub>1</sub> enzyme activity. In contrast, ATP inhibited G<sub>6</sub>PDH<sub>2</sub>, and its influence was specific. The inhibitory effect increased with increasing concentrations up to 0.4 mM (decrease in enzyme activity by about 36 %). A further increase in ATP concentration up to 1.2 mM decreased its inhibitory effect. At higher concentrations the inhibitory effect took place again, and at a concentration of 2.0 mM the G<sub>6</sub>PDH<sub>2</sub> activity decreased twofold (Fig. 8).

Thus, comparison of catalytic properties of cytosolic and plastidic isoforms of G<sub>6</sub>PDH indicated some differences, such as pH-optimum and regulation of activity by concentrations of substrate, NADPH, and ATP.

Table 1. Purification of isoforms of glucose-6-phosphate dehydrogenase from barley leaves.

Purification stage	Fraction volume [cm <sup>3</sup> ]	Total activity [nkat]	Protein content [mg]	Specific activity [mkat kmg <sup>-1</sup> (protein)]	Degree of purification	Activity yield [%]
Homogenate	30.0	144.50±13.83	405.90±3.44	0.36±0.03	1.00	100.00
Fractionation with (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (30-60% saturation)	2.5	114.02±9.17	100.65±8.62	1.13±0.17	3.14	78.90
Sephadex G-25	5.6	78.01±14.00	36.96 ± 3.48	2.11±0.17	5.86	53.90
Isoform I						
DEAE-cellulose	7.0	56.01±3.83	6.54±0.35	8.56±0.67	23.78	38.80
Sephadex G-150	6.0	8.17±1.33	0.22±0.02	38.00±1.50	105.56	5.70
Isoform II						
DEAE-cellulose	6.0	21.50±2.50	2.10±0.18	10.24±0.50	28.44	14.90
Sephadex G-150	6.0	3.83±1.00	0.17±0.01	22.52±1.33	65.56	2.70

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