# PARTIAL PURIFICATION AND PROPERTIES OF L-GLUTAMINE: D-FRUCTOSE 6-P AMIDOTRANSFERASE FROM HUMAN PLACENTA

M. Vessal and N. Gharai

*Department of Biochemistry, Shiraz University of Medical Sciences, Shiraz, Islamic Republic of Iran*

**Abstract**

The first enzyme of the pathway for uridine diphosphate N-acetyl-**D**-glucosamine (UDPAG) biosynthesis i.e. **L**-glutamine: **D**-fructose 6-P amidotransferase (**E.C.** 2.6.1.16) was purified 52-fold from human placenta using methanol fractionation and column chromatography on DEAE-Sephadex A-50. The enzyme showed optimal activity in a broad range of pH from 5.8 to 7.8 in both phosphate and cacodylate buffers. Its $K_m$ value for **D**-fructose 6-P was found to be 2.14 mM. The enzyme was inhibited up to 76% in the presence of 0.12 mM UDPAG. A $K_i$ value of 6.6 μM was obtained for the feedback inhibition of this enzyme by UDPAG.

## Introduction

The enzyme, **L**-glutamine: **D**-fructose 6-P amidotransferase (**E.C.** 2.6.1.16) which is the first enzyme of a pathway for the biosynthesis of uridine diphosphate-N-acetyl-**D**-glucosamine (UDPAG) catalyzes the biosynthesis of **D**-glucosamine 6-P from **D**-fructose 6-P and **L**-glutamine. This enzyme is located at a branch point in carbohydrate metabolism and competes for fructose 6-P with other pathways utilizing hexose monophosphates such as glycolysis, glycogenesis and hexose monophosphate shunt. The amidotransferase enzyme is subject to end product inhibition by UDPAG and appears to be the rate limiting enzyme in hexosamine biosynthesis [1].

**L**-glutamine: **D**-fructose 6-P amidotransferase has been partially purified from several animal and plant sources and its kinetic properties studied [2,3,4,5,6,7]. In a study of the distribution of this enzyme in different rat organs, placenta, other fetal tissues and the tissues involved in mucopolysaccharide secretion were found to have the highest concentration of this enzyme [8]. Hexosamines, aminosugar constituents of several glycoproteins and proteoglycans, have been detected in human placenta [9]. It has also been reported that human placental slices undergo hexosamine biosynthesis in media containing **D**-glucose and **L**-glutamine [10]. In this investigation human placenta was chosen as a readily available human organ with fairly high amidotransferase activity in order to partially purify this enzyme. Studies on the kinetic properties of this enzyme including its feedback inhibition by UDPAG has also been undertaken. The kinetic properties of this enzyme are compared to those of the enzymes from other sources.

**Keywords:** Human placenta, L-glutamine: D-fructose 6-P amidotransferase, purification, kinetic studies

## Materials and Methods

### Materials

**L**-glutamine, ethylenediaminetetracetic acid (EDTA, disodium salt), and dimethylarsonic acid (cacodylic acid) were purchased from BDH Chemicals (Poole, England). **D**-glucosamine 6-phosphate (Gm-6-P, disodium salt), **D**-fructose 6-phosphate (F-6-P, disodium salt), and uridine diphosphate N-acetyl-**D**-glucosamine (UDPAG, sodium salt) were obtained from Sigma Chemical Co. (St. Louis, Mo). DEAE-Sephadex A-50 was the product of Pharmacia, Sweden. All other reagents were of

analytical grade.

A "buffer mixture" based on that used by Ghosh *et al.* [4] was frequently employed throughout the experiment. It contained the following reagents in μmoles per ml: L-glutamine, 150; potassium phosphate buffer (pH 7.3), 10; and EDTA-disodium salt, 2.0 (final pH adjusted to 7.3 by dropwise addition of 45% KOH).

## Methods

**Enzyme Assay.** The standard incubation mixture for the assay of the enzyme included the following reagents in μmoles in a final volume of 0.5 ml; F-6-P, 2.0; L-glutamine, 15; potassium phosphate buffer (pH 7.7), 30; EDTA (disodium salt), 1.2; and 0.1 ml of the enzyme solution. The final pH of the incubation mixture before the addition of the enzyme was 7.3. The mixture was incubated at 37°C for 1 hr. The reaction was stopped by heating the tubes in a boiling water bath for 2 min. After cooling and centrifugation at 27,000 X g for 5 min at 0°C, 0.25 ml of the supernatant solution was analyzed for Gm-6-P [6]. Enzyme unit is expressed as μmoles of Gm-6-P produced per hr per 0.5 ml of the incubation mixture under standard assay conditions at 37°C. Protein was determined by the method of Lowry *et al.* [11].

**Preparation of Crude Enzyme.** A portion of fresh normal human placenta (135 g), obtained from Shiraz Maternity Hospital, was cut into small pieces and washed several times with cold 0.05 M potassium phosphate buffer (pH 6.6) containing 2.0 mM EDTA. The washed pieces were homogenized with 100 ml of the same buffer in a precooled Waring blender for 3 cycles of 40 sec each. The homogenate was centrifuged at 27,000 X g for 20 min at 0°C and the supernatant solution containing between 30 to 40 mg protein per ml was immediately employed for the purification of the enzyme.

**Methanol Fractionation.** To 15 ml of the crude extract, 5 ml of cold methanol (-20°C) were gradually added, while the enzyme solution was constantly being stirred. The turbid solution formed was centrifuged at 27,000 X g for 10 min at -15°C. To the entire resulting

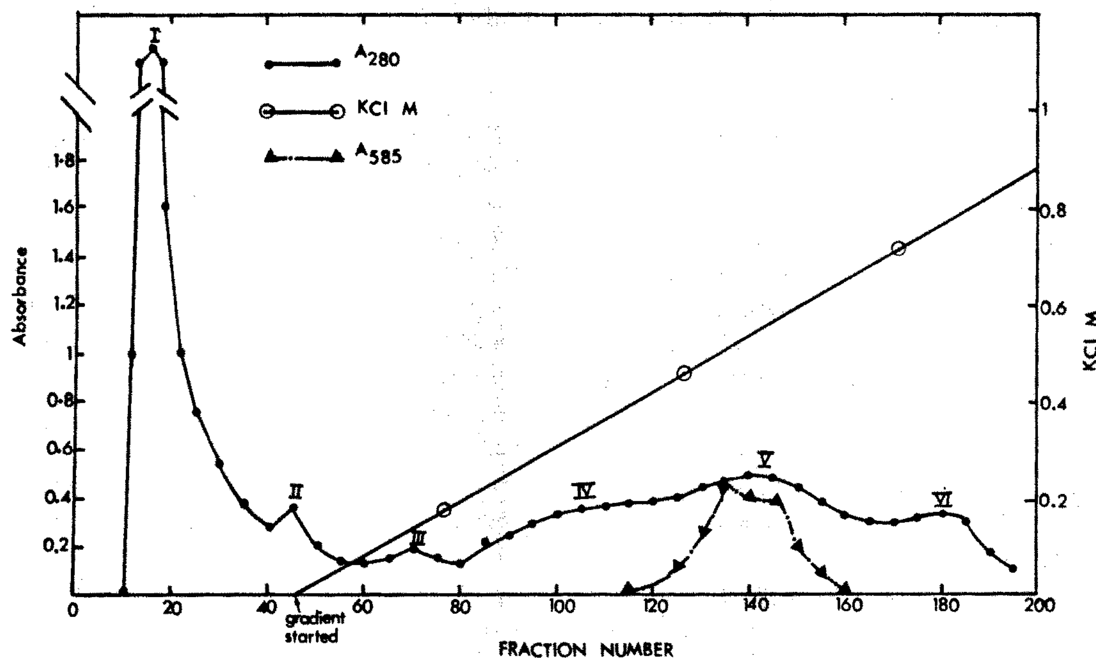


**Figure 1.** A typical DEAE-Sephadex A-50 column chromatogram of human placental proteins. Chromatography was performed as described under "Methods". The enzyme was eluted with linear KCl gradient (peak V). $A_{585}$ (- Δ-Δ -) refers to the colorimetric method of enzyme activity measurement and $A_{280}$ (- o-o -) indicates protein elution pattern.

supernatant solution, another 5 ml of cold methanol (-20°C) were gradually added, and the turbid solution was again centrifuged as before. The precipitate formed was suspended in 2.0 ml of the "buffer mixture" and left overnight at -20°C. Overnight freezing and subsequent thawing resulted in increased specific activity of the enzyme fraction [6]. The suspension was then centrifuged at 27,000 X g for 10 min at 0°C; the supernatant solution is referred to as the "methanol fraction".

**DEAE-Sephadex A-50 Column Chromatography.** DEAE-Sephadex A-50 (Pharmacia, Sweden) was equilibrated with an excess of the "buffer mixture" (pH 7.3). A column (35 x 2.5 cm) was packed with the DEAE-Sephadex slurry and the column was washed with the "buffer mixture" until the pH of its eluate was 7.3. Twelve milliliters of the methanol fraction (about 40 mg protein per ml) was applied to the column and eluted with the same "buffer mixture". Five milliliter fractions were collected. The resulting eluate gave a large protein peak as shown in Fig. 1 (peak I). The elution was continued with the aid of a linear salt gradient by using two identical containers each containing 500 ml "buffer mixture" (pH 7.3) to one of which 1 M KCl was added. The protein elution pattern was established by reading the absorbance at 280 nm against the buffer mixture. Different protein peaks were pooled separately and concentrated over a PM-10 Diaflo membrane (Amicon Corporation) under nitrogen gas. The solution from each concentrated peak was assayed for enzyme activity under standard assay conditions and the fraction containing the enzyme (Fig. 1, peak V) was stored at -20°C prior to its use in kinetic studies.

## Results

**Enzyme Purification.** A typical DEAE-Sephadex chromatogram of the enzyme is shown in Fig. 1, and a summary of the data on the partial purification of the enzyme is presented in Table I. A 52-fold purification of the enzyme was obtained. The enzyme was stable at -20°C for about a week with a gradual decrease in activity afterwards.

**Effects of Time, Enzyme Concentration and pH on Enzyme Activity.** Fig. 2 demonstrates the time course of the partially purified enzyme. The effects of enzyme concentration and pH on enzyme activity are shown in Figs. 3 and 4, respectively. The reaction follows a straight line up to 75 min tested (Fig. 2) and the activity is linearly proportional to protein concentration up to 275 µg of protein used per 0.5 ml of the incubation mixture (Fig. 3). The partially purified enzyme is active at a pH range of 5.8 to 7.8 and that the replacement of phosphate buffer by cacodylate buffer inhibits the enzyme activity by 50% (Fig. 4).

**Kinetics of L-Glutamine: D-Fructose 6-P Amidotransferase.** The initial velocity of the amidotransferase as a function of F-6-P concentration at a fixed concentration of L-glutamine (30 mM) together with the Lineweaver-Burk double reciprocal plot is

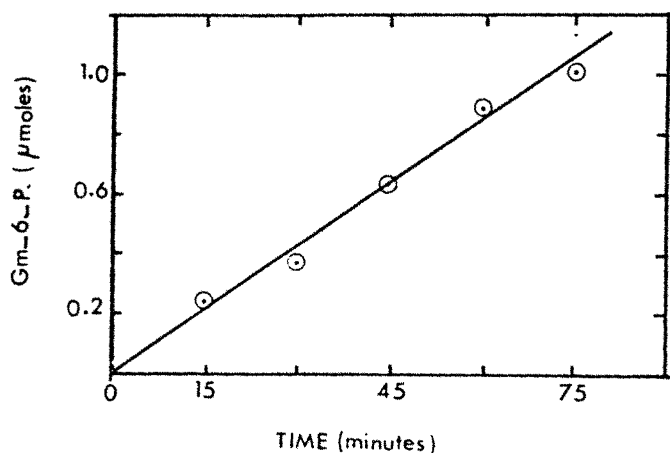


**Figure 2.** Enzyme activity as a function of time. The DEAE-Sephadex fraction (137 µg protein) was assayed under standard conditions except that the reactions were stopped at time intervals indicated. Results expressed as µmoles of Gm-6-P formed per 0.5 ml of the incubation mixture.

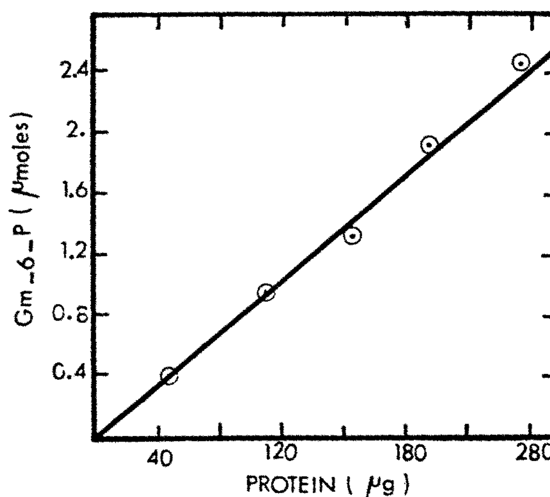


**Figure 3.** The effect of protein concentration on enzyme activity. Assays were done under standard conditions, except that the protein concentration was varied as indicated. The results are expressed in terms of enzyme units.

shown in Fig. 5, A and B. Double reciprocal plot was obtained upon calculation of the equation of the regression line [12]. Maximum enzyme activity was demonstrated at a concentration of 14 µmoles of F-6-P per ml. The enzyme was inhibited at higher concentration of F-6-P (Fig. 5A). A$K_m$ value of 2.14 mM was calculated for **D**-fructose 6-P from the X-intercept of the regression line.

Due to the instability of the enzyme in the absence of **L**-glutamine and our inability to remove **L**-glutamine and replace it by F-6-P without complete loss of enzyme activity [6], the $K_m$ value of the enzyme for **L**-glutamine could not be calculated.

The inhibition pattern of the enzyme by UDPAG is shown in Fig. 6, and a Dixon plot [13] showing the type of inhibition is demonstrated in Fig. 7. As shown, the enzyme is inhibited up to 76% by 0.12 mM concentration of UDPAG (Fig. 6); a $K_i$ value of 6.6 µM was obtained for the inhibitor acting competitively with respect to F-6-P (Fig. 7).

## Discussion

The specific activity of the crude placental **L**-glutamine: **D**-fructose 6-P amidotransferase (0.076 units per mg protein, Table I) is higher than the values of 0.02 to 0.046 units per mg protein obtained for fairly similar fractions from rat liver [1,4] but is identical to

**Table I.** Purification of **L**-glutamine: **D**-fructose 6-P amidotransferase from human placenta.

| Step | Total Activity (units) | Total Protein (mg) | Specific Activity (units/mg) | Yield % | Purfication (Fold) |
|---|---|---|---|---|---|
| 1. Crude | 880.9 | 11631.7 | 0.076 | 100 | 1 |
| 2. Methanol Fraction | 667.0 | 470.0 | 1.419 | 75.7 | 18.7 |
| 3. DEAE-Sephadex A-50 | 165.0 | 42.1 | 3.919 | 18.7 | 51.6 |

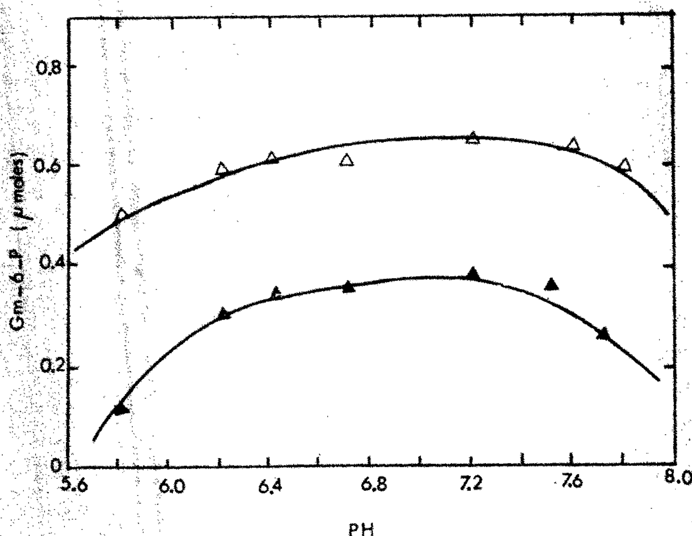


**Figure 4.** Enzyme activity as a function of pH. Assays were done under standard conditions except that the pH of the incubation mixtures was varied as indicated. The buffers were either 30 µmoles of potassium phosphate buffer at the indicated pH (- Δ-Δ -) or 30 µmoles of sodium cacoldylate buffer at the given pH (- ▲-▲ -). The enzyme source was 137µg of DEAE-Sephadex fraction. Results are in terms of enzyme units.

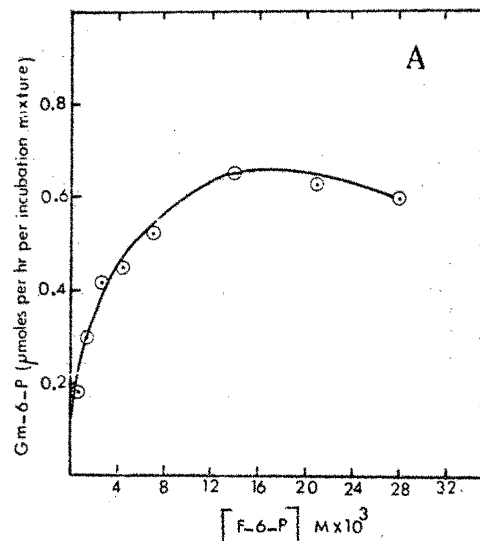


**Figure 5.** A.Rate of amidotransferase activity as a function of **D**-fructose 6-P concentration; B. The double reciprocal plot of A. The assays were done according to standard conditions except that **D**-fructose 6-P concentrations were varied as indicated. The pH of the incubation mixture was 7.3 and the enzyme source was 191 µg protein from DEAE-Sephadex fraction. Results are expressed in terms of enzyme units. The data are means of two separate experiments, which did not differ significantly.

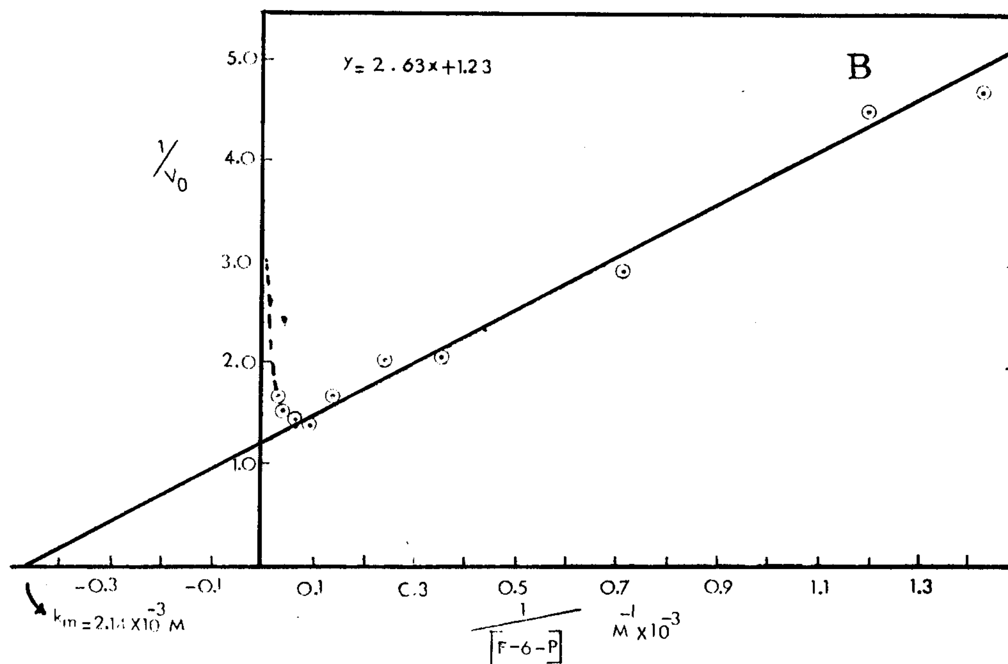


**Figure 5B.**

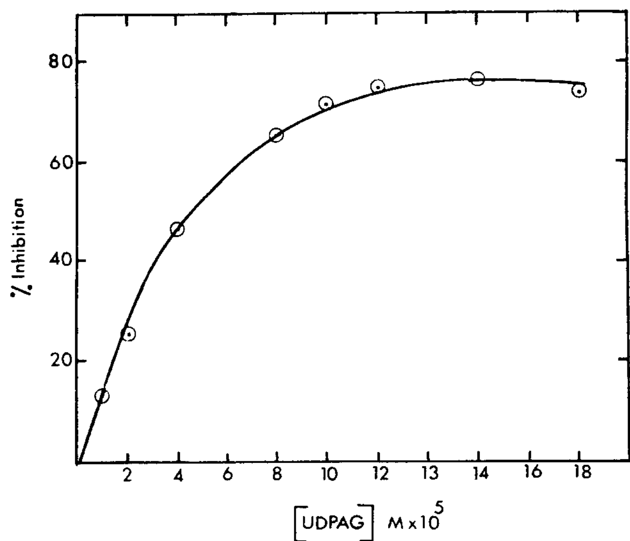


**Figure 6.** Inhibition pattern of the placental enzyme by UDPAG. Enzyme source was 191 μg protein from DEAE-Sephadax fraction. Assays done under standard conditions with the inclusion of the designated levels of UDPAG.

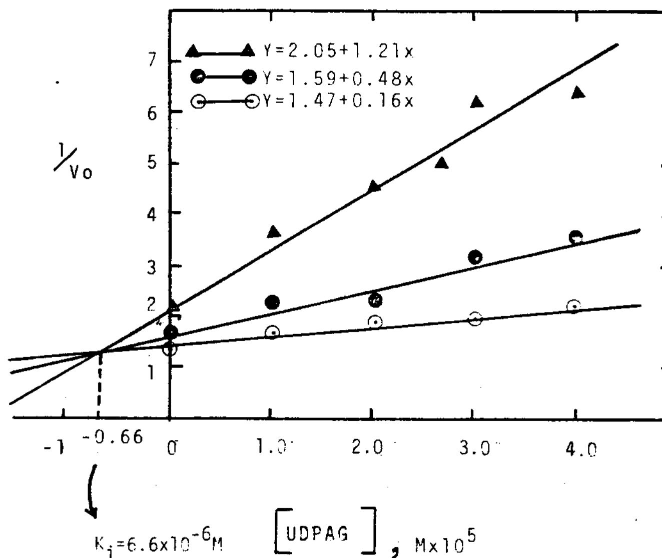


**Figure 7.** Competitive inhibition of placental amidotransferase by UDPAG. The incubation mixtures contained the same amount of reagents per 0.5 ml as the standard conditions except that D-fructose 6-P concentrations were 14 mM (- o-o -), 7 mM (o-o), and 2.8 mM (Δ-Δ) and UDPAG was included as indicated. The pH of the incubation mixture was 7.3. Enzyme source was 191 μg protein from DEAE-Sephadex fraction. Initial velocities expressed in terms of enzyme units.

the value of 0.078 units per mg protein reported for HeLa cells [1].

This enzyme was fairly unstable and difficult to purify by the ordinary fractionation techniques. Attempts were made to stabilize the enzyme using different reagents such as glycerol, sucrose, dimethyl sulfoxide, mercaptoethanol, and L-glutamine. Only L-glutamine at 0.15 M concentration was found to stablilize the enzyme to a much higher extent than the other reagents used. For this reason, all solutions employed in enzyme purification steps contained 0.15 M L-glutamine.

The activity of the enzyme in the pH range of 5.8 to 7.8 (Fig. 4) is comparable to the range reported by others for rat liver [1,4,5]. The $K_m$ value of 2.14 mM obtained for F-6-P (Fig. 5, A) is comparable to the value of 2.0 mM reported for *E. coli* [4], but is one order of magnitude higher than the value reported for the rat liver enzyme [1,4].

Feedback inhibition of the enzyme from other sources by UDPAG has been reported [1,6,14]. A 76% inhibition of the enzyme activity by 0.12 mM UDPAG (Fig. 6) is comparable to the respective values of 80, 74 and 78% obtained for the rat liver, mouse liver and HeLa cells by about 0.08 mM concentration of UDPAG [1]. The $K_i$ value of 6.6 μM (Fig. 7) obtained for the competitive inhibition of this enzyme by UDPAG is of the same order of magnitude as the values reported for the enzyme from other sources. The $K_i$ values reported for rat liver and the HeLa cells [1] were 5 and 11 μM, respectively; while that of the rat gastric mucosa was 1 μM, [7].

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