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A Rapid, Novel High Performance Liquid Chromatography Method for the Purification of Glutathione S-transferase: An Application to the Human Placental Enzyme

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A simple High Performance Liquid Chromatography procedure is detailed for the purification of Glutathione S-transferase. The human placental transferase was used to assess its potential. Unlike conventional methods of purification, the procedure is rapid and resolution of the various forms is achieved in less than 20 min. Since recovery is essentially complete, it is possible to isolate different minor forms. Three forms, one major and two minor, were separated. The major form represented about 97% of the total recovered activity and exhibited a specific activity of 254.94 μmoles/min/mg protein with a purification of 1342-fold. Electrophoresis of the major form revealed the presence of a single band, suggesting homogeneity.

GSHTr are a group of enzymes that catalyze the conjugation of GSH with numerous electrophiles in the initial step of mercapturic acid formation (1). The importance of GSHTr in the activation and detoxification of various xenobiotics is well established (2). The presence of different isozymes in various tissues is extensively documented. Jakoby et al. (3) have recently recommended that each isozyme be characterized biochemically.

Current procedures for the purification of GSHTr rely on a series of column chromatographic techniques and other methods, such as ammmonium sulfate precipitation, and IEF. However, these methods are tedious and inefficient. For example, DEAE-cellulose chromatography requires up to 38 hrs and results in only 10 - 53% recovery of enzyme (4-8). Gel filtration, CM-cellulose

The following abbreviations were used: HPLC, High Performance Liquid Chromatography; GSHTr, Glutathione S-transferase(s) (E.C. 2.5.1.18); IEF, isoelectric focusing; CDNB, 1-Chloro-2,4-dinitrobenzene; GSH, reduced glutathione; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

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chromatography, and chromatofocusing each require approximately 13 - 18 hrs and yield 34 - 95%, 59%, and 40% recovery, respectively (4-7,9-11), while IEF may take up to 96 hr and results in only 10% recovery (6,8,12). A possible concomitant loss of certain labile forms, due to poor recovery, is another aspect which has not been adequately addressed.

Although Vander Jagt et al. (13) reported a recovery of 85 - 95% with affinity chormatography, this procedure cannot resolve different isozymes of GSHTr. The presence of subpopulations of specific isozymes has been shown. However, due to lack of appropriate methodology, detailed characterization was not possible (4,9).

In light of the shortcomings of previously used techniques, we herein describe a HPLC procedure which is rapid, offers high resolution, and results in complete recovery of enzymatic activity. Human placental GSHTr was selected to evaluate its applicability, since the presence of a single enzyme has been reported (6,8,13-15).

MATERIALS AND METHODS

CDNB was purchased from Aldrich (Milwaukee, WI.). GSH and DTT were obtained from United States Biochemical Co. (Cleveland, OH.). The Sepharose 4B, and 2-mercaptoethanol were from Sigma Chemical Co. (St. Louis, MO.). All other chemicals used were of ACS grade and obtained commercially.

Enzyme Assay

GSHTr activity was determined spectrophotometrically, using an Aminco DW-2, according to the method of Vander Jagt et al. (13), with slight modification. The reaction mixture consisted of 1 mM CDNB, 2.5 mM GSH, 0.1 M potassium phosphate buffer, pH 6.5, and a rate-limiting quantity of enzyme in 3 ml. After a 3 min pre-incubation at 37 °C, the reaction was initiated by the addition of CDNB. Incubation mixture without enzyme served as control. The linear increase in absorbance at 340 nm was monitored at 37 °C. One enzyme unit (U) is defined as 1 µmole of CDNB-conjugate formed per min (extinction coefficient of 9.6/mM/cm). Specific activity is reported as U per mg protein. Protein content of the placental cytosol was determined according to method of Lowry et al. (16), while the dye binding assay of Bradford (17) was used in subsequent purification steps.

Enzyme Isolation

Term placentas from healthy women were obtained immediately after parturition and processed within one hour. The following procedures were carried-out at 4 °C unless specified otherwise. Chorionic tissue (150 g) was teased free of connective tissue, washed copiously with 50 mM Tris buffer containing 0.25 M sucrose, pH 7.4 (Buffer A) to remove residual blood components, and homogenized in 750 ml of Buffer A using a Waring blender. The homogenate was centrifuged at 12,250 X g for 20 min and the resulting supernatant was then centrifuged at 100,000 X g for 60 min. The post-

microsomal supernatant served as the starting material for affinity chromatography.

Affinity Chromatography

The epoxy-activated Sepharose 4B was synthesized according to the method of Porath (18) and the GSH was coupled to the activated 4B as described by Simons and Vander Jagt (19). In a typical experiment, approximately 200 U were applied to the 4B-GSH column (2.8 X 8 cm), which was previously equilibrated with 50 mM Tris buffer, pH 7.4 at 4°C (Buffer B). Flow rate was maintained at 0.5 ml/min. The column was then washed with Buffer B until no protein was detectable in the effluent. The GSHTr was eluted from the matrix with 50 mM Tris buffer containing 10 mM GSH, pH 9.6 at 4 °C and collected in 1 ml fractions. Those fractions exhibiting GSHTr activity were pooled and the pH was adjusted to 7.4 with 1 M potassium phosphate monobasic.

High Performance Liquid Chromatography

The HPLC system consisted of an Altex model 420 microprocessor with two Isco model 825 metering pumps, a Rheodyne model 7125 sample injector, and a Spectro-Monitor III variable UV detector set at 280 nm. The mobile phase consisted of 5.5 mM citrate potassium phosphate buffer containing 1 mM DTT and 10 mM GSH, pH 5.85 (Buffer C) and Buffer D containing 0.4 M KCl in Buffer C. Prior to chromatography the 4B-GSH eluent was concentrated using a PM-10 membrane, and then dialyzed overnight against 10 mM potassium phosphate buffer containing 10 mM GSH, pH 6.0. An aliquot (0.1 - 2 ml) was injected onto the anion exchange column (Synchrom AX-300 from Synchrom, Linden, IN.) and eluted with a salt gradient. The program was as follows: flow rate of 1 ml/min and % Buffer D = 0 at time 0; % Buffer D was increased to 7.5 in 3 min, beginning at time 5 min; % Buffer D was further increased to 100 in 0.1 min at 8 min; and re-equilibration began at 16 min, at which time % Buffer D was decreased to 0 over 2 min and flow (1.5 ml/min) was maintained for 15 min. The HPLC effluent was collected in 1 ml fractions and assayed for GSHTr activity. Those fractions exhibiting distinct GSHTr activity peaks were combined and specific activity was determined.

Electrophoresis

PAGE was performed according to the method of Davis (20), with slight modification. Included in the running buffer was 10 mM GSH. A current of 25 mA/gel was applied. Gels were fixed overnight in isopropanol : glacial acetic acid : water (25:10:65, v/v) and stained in a 0.05 % solution of Coomassie Blue R-250 in isopropanol : glacial acetic acid : water (25:10:65, v/v) for 3 hrs. All gels were destained by diffusion using 7 % glacial acetic acid.

RESULTS

Table 1 summarizes the results of the purification of GSHTr from five term placentas, using the two step procedure. Affinity chromatography resulted in substantial purification (533-fold) with a recovery of 97% (Table 1). These results are similar to those reported by Vander Jagt *et al.* (13). Although this initial step eliminates hemoglobin, a known inhibitor (14), it does not yield a homogeneous GSHTr preparation. The presence of several proteins bands upon PAGE (Fig. 1) suggests that various proteins are adsorbed by the affinity matrix and co-elute with placental GSHTr.

Table 1: Purification of Human Placental GSHTr

Fraction	Specific Activity* (μmoles/min/mg)	Purification	Recovery (%)
Supernatant	0.19 ± 0.02	1	100
4B-GSH	101.29 ± 13.88	533	97
HPLC	254.94 ± 17.34**	1342	124

*Values reported are mean ± S.E.M. (n=5)
**$p<0.005$ as compared to 4B-GSH

Our preliminary experiments on the optimization of HPLC conditions indicated that the inclusion of various protective agents, such as GSH and DTT, in the elution buffers was essential for retention of biological activity and acceptable yield of GSHTr. In the absence of such agents, the enzyme recovery was only 2.7% of the concentrated 4B-GSH applied. With the inclusion of GSH, recovery increased to approximately 75%. Addition of 1 mM mercaptoethanol, 5% glycerol, or 1 mM DTT to the elution buffers in order to supplement the 10 mM GSH present resulted in 49%, 83%, and 111% recovery of

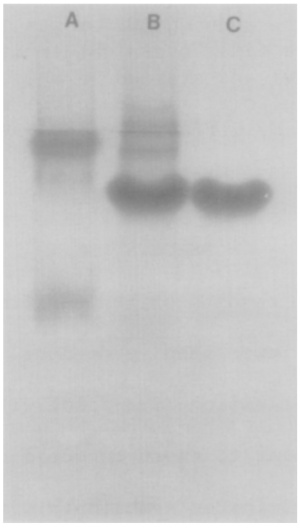

Figure 1: Polyacrylamide Gel Electrophoresis of Human Placental Glutathione S-transferase

100 μg of protein of each fraction was applied. A, 100,000 X g supernatant; B, concentrated 4B-GSH eluent; C, concentrated HPLC P 2.

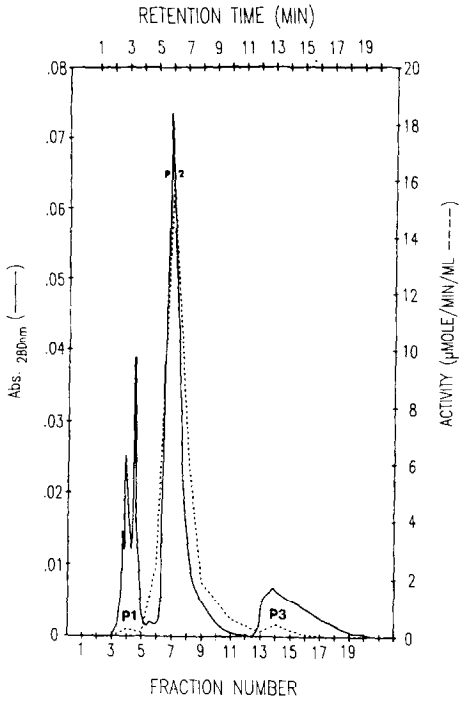

Figure 2: High Performance Liquid Chromatographic Separation of Human Placental Glutathione S-transferase

154 μg of concentrated 4B-GSH eluent was applied to the Synchrom AX-300 column. See Material and Methods for details.

the applied activity, respectively. Therefore, all subsequent experiments were performed using 10 mM GSH and 1 mM DTT in the mobile phase.

The GSHTr present in the 4B-GSH concentrate were resolved into 3 distinct, active peaks by HPLC. The chromatographic profile obtained from a typical experiment is depicted in Figure 2. About 97% of the recovered GSHTr activity was present in the major peak (P 2), with a retention time of 5.8 min. The observed average specific activity of 255 U/mg protein for P 2 represents an overall purification of 1342-fold (Table 1). Two additional minor peaks with retention times of about 3.0 (P 1) and 13.4 (P 3) min were consistantly observed. They represented approximately 1% (P 1) and 2% (P 3) of the activity.

The retention times (mean $\pm$ S.E., n=5) of 3.23 $\pm$ 0.43, 6.00 $\pm$ 0.43, and 13.96 $\pm$ 0.17 min were observed for P 1, P 2, and P 3, respectively. Re-chromatography of fraction 6 of P 2 (Fig. 2), under identical conditions,

yielded a peak with a retention time of 5.8 min, containing 99% of the activity, demonstrating that HPLC separation was reproducible.

In contrast to the presence of six bands in the concentrated 4B-GSH preparation (Fig. 1B), a single band was observed with the concentrated HPLC P 2 fraction (Fig. 1C), suggesting homogeneity.

DISCUSSION

Several anomalies exist in the reported properties of highly purified human placental GSHTr. The values cited for the molecular weight of the dimer vary from 45,000 to 60,000 daltons (6,8,14,15,21,22) and that for the subunit range from 22,000 to 23,400 (13,15,21,22). A pI of 4.5 to 4.9 has been observed (6,8,11,14,15,21,22). This variability may, in part, be attributed to the possible heterogeneity of the preparations used. Differences in these parameters may also arise from different proportions of multiple conformers (13,14) present.

The presence of 3 distinct peaks of GSHTr activity (Fig. 2) suggests that the placental transferase is comprised of multiple conformers. Earlier, Polidoro et al. (14) did report the existence of 2 interconvertible forms using PAGE; whereas, Vander Jagt et al. (13) demonstrated kinetically the existence of three comformational states. We believe the minor band that Polidoro et al. (14) observed corresponds to P 3 (Fig. 2), since both are relatively more anionic in nature. P 1 interacts minimally with the anion exchange resin and may be the cationic form reported by Koskelo (11) or another weakly anionic form of placental GSHTr. However, the possibility that this may be one of the two forms of erythrocyte GSHTr (23) cannot be discounted at this time. Further investigations are in progress to determine the physical and biochemical properties of P 2 and P 3.

The reported HPLC procedure requires less than 20 min and is highly reproducible. Up to 1 mg of concentrated 4B-GSH eluent (112 U) can be injected onto the column without any significant loss in resolution. Thus, the major advantages of the HPLC procedure are rapid separation, excellent

resolution, and complete recovery of enzymatic activity. We believe that HPLC, with the necessary minor modifications, can be used to resolve and quantitatively recover different isozymes as well as conformers of GSHTr from various tissues. The potential versatility of this technique is presently being evaluated in our laboratory.

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