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Isolation and characterization of rabbit liver glutathione s-transferase

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ABSTRACT

Background: Glutathione S-transferases (GSTs) are enzymes that detoxify harmful compounds by conjugating them with glutathione, their structure and catalytic mechanisms are crucial for understanding cellular defense and disease biomarker identification.

Objectives: The study aimed to purify and characterize GST from rabbit liver, understand its structure, composition, isoenzyme diversity, and catalytic properties.

Methods: Enzyme Purification: GST was purified from rabbit liver, resulting in a 22-fold increase in activity with a recovery of 78-80%.

Stability and Optimal Conditions: The enzyme exhibited optimal activity at pH 6.5 and remained heat stable. However, it lost 50% of its activity after eight days of storage at room temperature.

Isoenzyme Separation: Chromatofocusing was used to separate the enzyme into eight isoenzymes, with isoelectric points (pI) ranging from 7.96 to 9.6.

Activity Assays: Conjugation rates were measured for substrates such as reduced glutathione, 1,2-epoxy-3-(nitrophenoxy) propane, and ethacrynic acid.

Results: The study found that the major isoenzyme, with a pI of 8.6, accounted for over 94% of total GST activity and had strong detoxification potential. Eight isoenzymes were identified with distinct pI values, indicating functional diversity. The structural differences between rabbit and rat liver GSTs were observed, with rabbit liver GSTs being heterodimeric and rat liver GSTs heterotrimers. Cross-reactions between antibodies against rabbit and sheep liver indicated a link between the different isoenzymes.

Conclusion: The study reveals the purification and characterization of GST from rabbit liver, revealing its structural properties, subunit composition, and catalytic activity. It suggests functional diversity, potentially influencing detoxification, drug resistance, and disease biomarker research.

Keywords: Affinity Chromatography ,Chromatofocusing ,CDNB ,Environmental Pollution Control, Glutathione S-Transferases (GSTs) ,Sustainable Detoxification

Introduction

Glutathione S-transferases (GSTs, EC 2.5.1.18) are multifunctional enzymes that play a central role in the detoxification of a wide range of xenobiotics and endogenous compounds. These enzymes are indispensable for cellular defense mechanisms, contributing to protection against oxidative stress, inflammation, and tissue damage through both catalytic and non-catalytic activities (1, 2, 3). Catalytically, GSTs facilitate the conjugation of the tripeptide glutathione (GSH) to electrophilic substrates, thereby enhancing their solubility and promoting their excretion or neutralization (4). Beyond their catalytic functions, GSTs are involved in a variety of non-catalytic processes, including signal transduction, apoptosis, and cellular transport, highlighting their broad functional significance in maintaining cellular homeostasis (5, 6).

The protective role of GSTs against oxidative damage is well-established, as these enzymes serve as a primary defense mechanism against reactive oxygen species (ROS) and other free radicals generated during normal metabolic processes or in response to environmental toxins (7). This protective function is particularly critical in tissues such as the liver, which is frequently exposed to a multitude of endogenous and exogenous toxicants (8). Over the years, a diverse array of GST isozymes has been isolated from various species, including humans, rats, and mice, revealing significant functional diversity among these isoforms (9). These isozymes are classified into distinct classes based on their substrate specificity, immunological properties, and amino acid sequences, reflecting

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their evolutionary adaptation to diverse physiological roles (10).

Recent studies have focused on elucidating the similarities and differences in GST isozymes across mammalian species, particularly in the liver, where these enzymes are highly expressed. For example, Dai et al. (8) demonstrated notable interspecies variations in GST isoforms, with certain isozymes exhibiting unique affinities for specific substrates, including xenobiotics and endogenous molecules such as bilirubin. In rat liver cytosol, at least 12 electrophoretic forms of GST have been identified, each displaying distinct properties and substrate preferences (11, 12, 13, 14). This diversity suggests that gene duplication and subsequent divergence have played a significant role in the evolution of the GST superfamily, contributing to its functional complexity and adaptability (15).

The interaction of GSTs with bilirubin has garnered particular interest due to its potential implications in bilirubin metabolism and detoxification. Several studies have demonstrated that GSTs, particularly those in the liver, exhibit a strong affinity for bilirubin. Recent investigations suggest that bilirubin binding occurs not only at the catalytic site but also at other distinct regions of the enzyme, indicating a broader regulatory role for GSTs in bilirubin homeostasis (16, 4). These findings have significant therapeutic implications for conditions associated with bilirubin accumulation, such as jaundice and certain liver dysfunctions.

In this study, we report the purification and characterization of various glutathione S-transferase isoforms from rabbit liver, building on previous research conducted in other species. We provide evidence of immunological similarities among the electrophoretic forms of the enzyme, further advancing our understanding of GST diversity in non-human species. Additionally, we investigate the effects of specific chemical inhibitors on GST activity in rabbit liver, offering new insights into the modulation of GST function and its potential therapeutic applications in oxidative stress-related conditions. This work contributes to a deeper understanding of the structural and functional diversity of GSTs and their role in cellular detoxification and homeostasis.

Materials and Methods

Animals and Tissue

One-year-old white rabbits *(Lepus arabicus)* were obtained from the Yarmouk University animal house. Immediately after euthanasia, the liver was excised, placed on ice, and transported to the laboratory. The tissue was either processed immediately or stored at -20°C until further use. For antibody production, 4-month -old white rabbits raised and bred at the Yarmouk University animal house were utilized.

Chemicals

Reduced glutathione (GSH), reduced glutathione-linked agarose, gel filtration protein standards, SDS-electrophoresis protein standards, sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue R-250, Tris-base, bovine serum albumin (BSA), complete and incomplete Freund's adjuvants, sodium azide, β-mercaptoethanol, acrylamide, N,N'-ethylene bisacrylamide, and Sephadex G-100 were purchased from Sigma Chemical Company (MO, USA). 1-Chloro-2,4-dinitrobenzene (CDNB) and bromophenol blue were obtained from BDH Laboratory Reagents (England). Polybuffer Exchanger 94 and Polybuffer 74 were ordered from Pharmacia Fine Chemicals (Uppsala, Sweden). Tetramethylethylenediamine was purchased from Bethesda Research Laboratories (USA). Barbital buffer was obtained from Gelman (USA). The herbicide (oxadiazolone) was sourced from Supelco Inc. (Switzerland), and the insecticide (diazinon) was obtained from Société des Usines Chimiques Rhône-Poulenc (France). All other chemicals were of the highest available purity and obtained from commercial sources.

Tissue Extract Preparation

A 20 g portion of rabbit liver was finely chopped and homogenized in 0.1 M potassium phosphate buffer (pH 6.5) at a 1:2 (w/v) ratio using a Waring blender at maximum speed for one minute. All subsequent procedures were carried out at 0–4°C unless otherwise specified. The homogenate was centrifuged at 37,000 × g for 30 minutes using an MSE Europa 24M centrifuge. The supernatant was filtered through glass wool, and the pellet was discarded.

Assay of GST Activity

Glutathione S-transferase (GST) activity was measured spectrophotometrically using a Pye Unicam SP 8-400 spectrophotometer. The substrates used included 1-chloro-2,4-dinitrobenzene, 2,4-dichloronitrobenzene, p-nitrobenzyl chloride, p-nitrobenzoyl azide, ethacrynic acid, 1,2-epoxy-3-(p-nitrophenoxy)propane, and 5β-androstan-3,17-dione. Enzyme activity was expressed in units of μmol/min at 25°C, and specific activity was calculated as μmol/min/mg protein. Protein concentration was determined using the method of Lowry et al. (1951), with bovine serum albumin as the standard.

Purification of Glutathione S-Transferase

Reduced glutathione agarose beads were swollen in 0.1 M potassium phosphate buffer (pH 6.5) and packed into a 1 × 5 cm column. The column was equilibrated with the same buffer at a flow rate of 30 mL/hour. The supernatant (400 mg protein) from the tissue extract was applied to the column at a flow rate of 20 mL/hour. Non-specifically bound proteins were removed by washing the column with 100 mL of equilibration buffer containing 0.2 M NaCl. The enzyme was eluted using 50 mM Tris-HCl buffer (pH 9.6) containing 10 mM reduced glutathione. Fractions (2 mL each) with the highest enzyme activity were pooled, dialyzed against 40 volumes of 0.1 M potassium phosphate buffer (pH 6.5) for 14 hours, and concentrated using an Amicon PM 30 membrane.

Separation of Isoenzymes by Chromatofocusing

Chromatofocusing was performed according to the manufacturer's instructions (Pharmacia). The enzyme purified by affinity chromatography was concentrated to approximately 2 mL using an Amicon PM 30 membrane and applied to a chromatofocusing column (0.9 × 30 cm) packed with Polybuffer Exchanger 94. The column was pre-equilibrated with 0.025 M Tris-HCl buffer (pH 9.4), and the enzyme was eluted with 270 mL of Polybuffer 74 (diluted 1:10 with water, pH 4.0). Fractions (2 mL each) were assayed for enzyme activity, and the pH of each fraction was recorded immediately. Fractions with the highest activity were pooled and separated from the polybuffer using affinity chromatography.

Determination of Molecular Weight

The molecular weight of the GST subunits was estimated using 12% SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970). Protein standards included phosphorylase B (94,000 Da), bovine serum albumin (67,000 Da), egg albumin (43,000 Da), carbonic anhydrase (30,000 Da), soybean trypsin in-

hibitor (20,100 Da), and lactalbumin (14,400 Da).

Antibody Production

Antibodies against purified rabbit liver GST were produced in rabbits as described by Hunaiti and Kolattukudy (1984). Approximately 350 µg of the purified enzyme was emulsified with complete Freund's adjuvant (1:1 v/v) and injected subcutaneously into rabbits. Booster injections containing 125 µg of the enzyme emulsified with incomplete Freund's adjuvant were administered at two-week intervals. Two weeks after the second injection, the rabbits were bled, and the antiserum was collected by centrifugation at 4,000 × g for 10 minutes. The effect of the antiserum on enzyme activity was determined by immunotitration. Aliquots (20 µg) of the enzyme were incubated with 200 µL of antiserum at varying dilutions, and the residual enzyme activity was measured using 1-chloro-2,4-dinitrobenzene as the substrate.

Ouchterlony Double Immunodiffusion

Ouchterlony double immunodiffusion was performed on microscope slides using 1% agarose in 75 mM barbital buffer (pH 8.8) containing 0.02% sodium azide. Wells (2 or 4 mm in diameter) were punched into the gel, and 10 µL of crude rabbit liver extract was added to the outer wells, while 15 µL of antiserum was added to the center well. The slides were incubated in a humid chamber for 24–48 hours, washed with 0.9% NaCl, and stained with 0.1% Coomassie Brilliant Blue R-250 in 5% acetic acid.

Inhibition Study

The inhibitory effects of sodium azide, bromophenol blue, and bisacrylamide on rabbit liver GST were investigated. Stock solutions of the inhibitors were prepared in ethanol and used at final concentrations of 1 mM and 2 mM. The enzyme (20 µg) was pre-incubated with the inhibitors and 0.1 mM reduced glutathione

in 0.1 M potassium phosphate buffer (pH 6.5) for 10 minutes. The reaction was initiated by adding 1 mM 1-chloro-2,4-dinitrobenzene, and enzyme activity was measured as described above. Controls without inhibitors were included in each assay.

Results

Glutathione S-transferase activity in the rabbit liver

Crude extracts prepared from rabbit liver contained GST activity. The specific activity of the enzyme was evaluated using CDNB, the most commonly used substrate. It was found that rabbit liver had a specific activity of 4.13 µmole/min/mg.

Purification of glutathione S-transferase

Using glutathione-linked agarose, a fast purification .The process was used to extract the glutathione S-transferase enzyme from the liver tissue in order to learn more about the nature of the enzyme's expressed activity.

After the rabbit liver's 37,000 xg supernatant is directly added to the affinity column, all glutathione S-transferase activity was retained by the column but not the bulk of the proteins Figure 1.

The enzyme was successfully recovered in a single sharp peak by 10mM reduced glutathione in 50Mm Tris-HCl buffer pH 9.6 (Figure.2). This purification procedure resulted in over 78% recovery of the applied glutathione S-transferase activity from rabbit liver and about 22 purification fold Table1.

Following a 2-minute preincubation period with inhibitors and 1 mM GSH at room temperature, the enzyme (30 µg) in 3 ml of 0.1 M potassium phosphate buffer pH 6.5 was exposed to the reactions. 1 mM l-chloro-2,4-dinitrobenzene was then added.

Purified rabbit liver glutathione S-transferase had high specific activity. It appeared from the results that rabbit liver enzyme constituted about 4.6% of soluble protein. The elution pattern for the glutathione S-transferase from the liver tissue on the affinity chromatography is given in Figure 1.

Table 1. Glutathione S-transferase purification from rabbit liver.

Fraction	Total protei (mg)	Total activity µmole/min	Specific activity µmole/min/mg protein	Purification (fold)	Yield %
Supernatant	346	1430	4.13	1	100
Affinity chromatography	12.36	1120	90.61	22	78.3

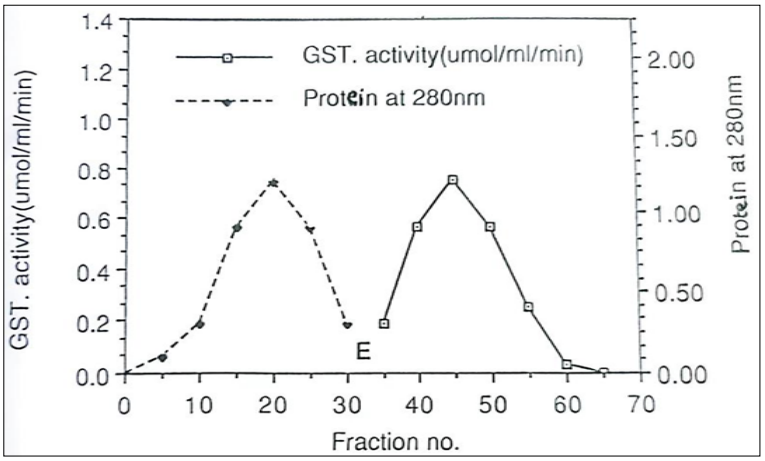

Figure 1. Purification of rabbit liver glutathione S-transferase by glutathione-linked agarose affinity chromatography. Protein was measured at 280nm after 1:40 dilution 0.1M potassium phosphate buffer pH 6.5.

Effect of pH on Glutathione S-transferase activity.

The effects of pH on the rate of glutathione conjugation with CDNB was studied for the liver purified glutathione S-transferase. In all cases below pH 5 the transferase activity was low and as the pH increased the rate of reaction increased reaching a pH optimum for the rabbit liver enzyme around 6.5 Figure 2.

Glutathione S-transferase subunit composition

12% SDS-polyacrylamide gel electrophoresis was used to assess the purity of the glutathione S-transferase that was extracted from the affinity column.

According to Figure 3. On a 12% SDS-polyacrylamide gel electrophoresis, the rabbit liver enzyme showed two bands of protein. These bands had molecular weights of 28,000 and 27,000, according to a linear plot of log molecular weight against standard protein mobilities Figure 3.

Resolution of the Isoenzymes

Eight peaks of activity were identified in the transferase using an isoelectric fractionation using Sephadex polybuffer exchanger 94 Figure 4. At a pH of approximately 8.7, the majority of rabbit liver GST isozymes showed strong activity.

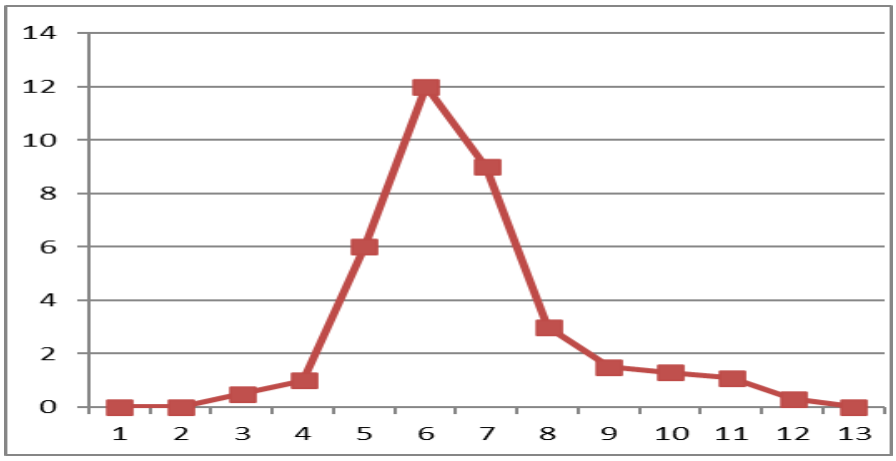

Figure 2. The effects of pH on glutathione S-transferase activity in the liver of rabbit.

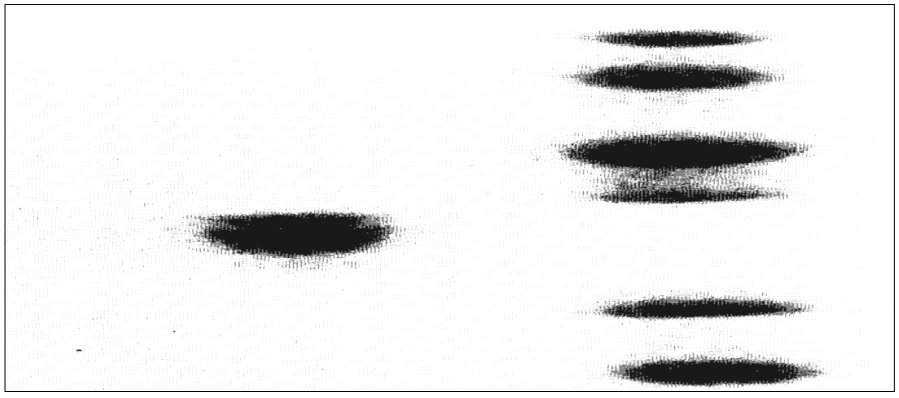

Figure 3. Glutathione S-transferase from purified rabbit liver separated by 12% SDS-gel electrophoresis. Standard protein indicators are located in Lane 1 and pure rabbit liver enzyme is located in Lane 2.

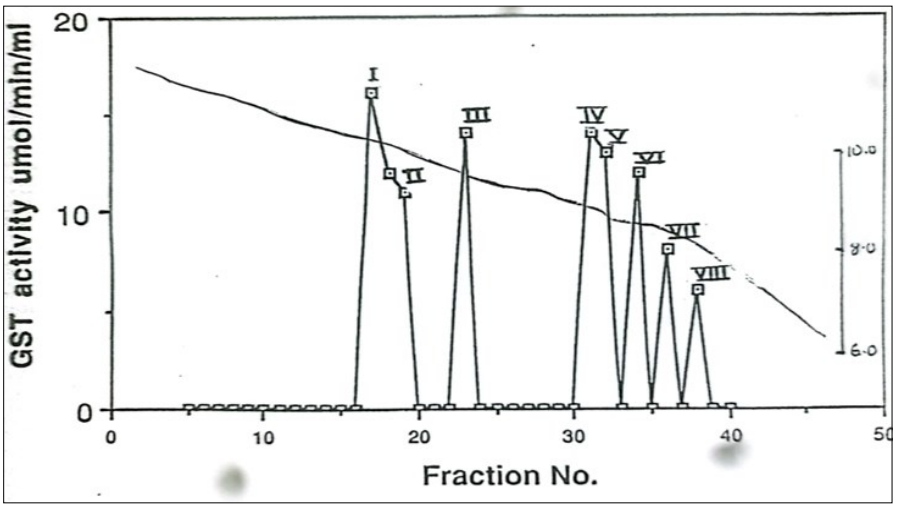

Figure 4. Purification of various isoenzymes of rabbit liver glutathione S-transferase by chromatofocusing Peak1 pI 9.6), Peak II(pI 9.2) , Peak III (pI 8.6) , Peak IV (pI 8.3), (Peak V (pI 8.1), Peak VI (pI 8.05), Peak VII(7.8),Peak VIII(7.5).

Substrate specificity

The main liver isoenzyme of rabbits (pI 8.4) showed maximum conjugation rates with GSH when 1–chloro-2,4-dinitrobenzene was used as a substrate Table 2. However, it also utilized alternative substrates at lower efficiency. For ethacrynic and 1,2-epoxy-3-(p-nitrophenoxy)propane, no appreciable rate was observed. The other isoenzymes' substrate specificity was not determined because of their limited availability.

Catalytic properties:

For a minimum of 7 minutes and 10 µg of protein per mL, the enhanced enzyme's quantifiable activity was linear. The subsequent studies employed only the initial velocities and were all conducted within the linear range of time and protein.

Ouchterlony:

The Ouchterlony double-diffusion research Revealed the presence of the rabbit antibody Produced against the principal peak (pI 8.7) of the chromatofocusing column. Figure 5.

Inhibitors

The rabbit enzyme was employed to study inhibitors from several sources that are known to impact GSH-S-transferases Table 3. The thiol-directed reagent iodoacetamide and the probable substrate N-(4-bromobutyl) phthalimide significantly reduced the enzyme activity, although the other reagents showed inhibition at relatively high doses Table 3.

The antigen, which can be an unprocessed or pure enzyme, generated a single precipitin line Figure 5. Similarly, chromatofocusing separated the remaining six enzyme peaks, and each of those peaks produced a single precipitin line from the antibody. The chromatofocusing-obtained eight electrophoretic forms show no spurs and completely merged immunoprecipitation lines, suggesting that they are immunologically quite similar.

they relate to one another. All eight may have diverged from a single gene from a common ancestor. In that instance, the antigenic characteristic was preserved. Reddy et al. noted that isoenzyme C-4 in sheep liver and GSH-transferase in rabbit liver have a large variance, despite the paucity of data. The two kinds of enzymes differ somewhat from one another in terms of their sizes, isoelectric points, and substrate specificities.

Isoelectrophoretic fractionation of rabbit liver GSH-transferases produced a well-defined pattern of isoenzyme distribution. Generally speaking, rabbit liver GST isozymes are highly active at pI of 8.7 Figure 4. Due to its strong binding affinity through hydrophobic, aromatic, and electrostatic interactions, structural compatibility with the enzyme's active site as demonstrated in Table 3, and capacity to induce conformational changes that lower enzymatic activity, 2 mM of bromophenol blue (BPB) is an effective.

Table 2. Substrate specificity of the primary glutathione S-transferase isoenzyme of rabbit liver.

Substrate	Concentration (mM)	Rabbit Liver	% relative activity
1-chloro-2,4-dinitrobenzene	1.0	100	100
1,2-epoxy-3-nitrophenoxy) propane	5.0	6.8	18
Ethacrynic	0.2	0.95	17.5

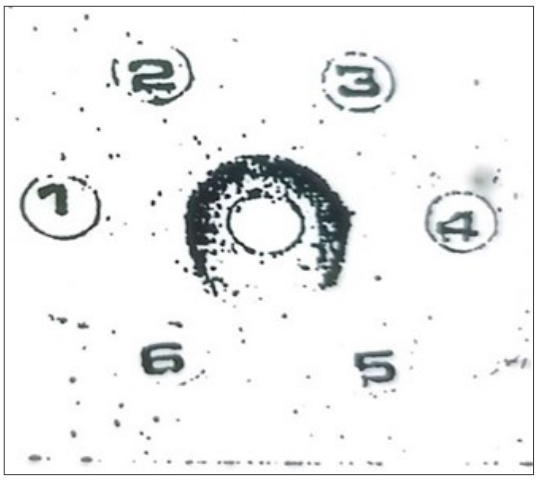

Figure 5. Analysis of double diffusion in was present in the center well in amounts of 20µl, whereas the purified isoenzyme was found in increasing amounts in the outside wells 1, 2, 3, 4, 5, and 6.Ouchterlony. Rabbit antiserum produced against the primary isoenzyme (pI 8.7).

Table 3. Inhibitors' effects on glutathione S-transferase in pure rabbit liver.

Inhibitors	(%) of Rabbit liver GST inhibition
Sodium azide 1 mM	40.8
Sodium azide 2 mM	63.6
Bromophenol blue 1 mM	79.5
Bromophenol blue 2 mM	84.3
Bisacrylamide 1mM	33.4
Bisacrylamide 2mM	53.2

Discussion

This paper first described the process of purifying glutathione S-transferase from rabbit liver, which enabled us to compare the rabbit liver enzyme with those from other mammalian sources (1, 2, 3, 4). The GSH-S-transferases identified in rabbits and rat liver (5, 6) differ in their subunit makeup. Rat liver enzymes are heterotrimers made up of subunits with molecular weights of 22,000, 23,500, and 25,000. On the other hand, rabbit GSH-S-transferases are heterodimeric proteins with subunits of molecular weights 28,000 and 27,000, respectively, and native molecular weights of approximately 54,000 and 53,000.

The subunit composition of rabbit enzymes is fairly similar to that of the human transferases currently in use as well as the GSH-transferases that have been isolated from sheep and human liver (7, 8, 9). With subunit molecular weights ranging from 22,500 to 26,000, these enzymes are heterodimers (10, 3). One interesting characteristic of glutathione S-transferase, which was isolated from the livers of various mammals, is the fact that it has multiple isozymes that can conjugate GSH with distinct electrophiles. Comparable to this, rabbit liver contains the enzyme in at least eight different electrophoretic forms (11).

Chromatofocusing approaches can be utilized to separate these isoenzymes, which may not be effective in ion exchange chromatography, as described for other mammalian sources. The immunological relationship between rabbit liver enzymes and human liver isoenzymes is similar, as demonstrated by the fact that antibodies raised against the major electrophoretic form (pI 8.7) of the rabbit enzymes cross-reacted with all eight forms of the enzyme (12, 7, 13). This is in contrast to liver enzymes from rats or mice. If the eight transferases present in rabbit liver exist at all, it is unclear how inhibitor of glutathione S-transferase. Because of this, BPB is an invaluable tool for studies and potential therapeutic uses involving GST inhibition (14). Even yet, great care was taken to wash the chromatofocusing column with a lot of salt. It is currently unclear how the eight different variants of rabbit GSH-transferase differ from one another in terms of substrate specificities and electrophile inducibility. Nevertheless, the current findings detail the first purification and division of the several GSH-transferase forms from rabbit tissue (15, 16, 17).

Ethics approval and consent to participate

The faculty of Nursing research ethics committee has approved the research proposal of Dr. Ahmad Al-Hadidi, number: IRB 0005 (17-2-2025).

Availability of data and materials

The raw data required to reproduce these findings are available in the body and illustrations of this manuscript are available in the body and illustrations of this manuscript.

Author's contribution

The first version of the document was written by Al Hadidi. Hunaiti was in charge of overseeing the organization of research activities and the primary conceptual ideas. Al Hadidi and Hunaiti were the ones who carried out the experiment. Explanation of the data, experimental design, and outcomes Al Hadidi and Hunaiti. Data curation and manuscript editing were done by Al Hadidi. The final draft of the work was reviewed by the authors, who also gave their approval.

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Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this article

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