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Purification and Characterization of Glutathione S-Transferase from rabbit liver

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Abstract: Glutathione S-transferases (GSTs) are essential enzymes involved in the detoxification process in animals. They catalyze the conjugation of the antioxidant glutathione (GSH) to various electrophilic compounds, such as environmental toxins, carcinogens, and metabolic by-products, forming mercapturic acids that are more water-soluble and can be excreted. This process protects cells from oxidative stress and chemical damage, and GSTs are particularly abundant in detoxification organs like the liver, kidneys, and lungs. In addition to detoxification, GSTs regulate cellular processes like signal transduction, apoptosis, and cell proliferation. GSTs were purified from rabbit liver with a 22-fold purification and 78-80% yield. The enzyme activity was assessed using 1-chloro-2,4-dinitrobenzene as a substrate, resulting in a specific activity of 91 µmole/min/mg protein. Gel filtration was performed on a Sephadex G-100 column to reveal the enzyme's native molecular weight of approximately 50,000. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was used to examine the enzyme's subunit composition, and its isoelectric points (pI) were determined using chromatofocusing. The purified GST enzyme from rabbit liver exhibited two distinct subunits with molecular weights of 28,000 and 27,000, and all enzyme activity was associated with a single protein band in native polyacrylamide gel electrophoresis. The enzyme displayed a pH optimum around 6.5 and was minimally affected by heat, with 50% of activity retained after eight days of storage at room temperature. The enzyme showed higher conjugation rates for reduced glutathione with substrates like 1,2-epoxy-3-(nitro phenoxy) propane and ethacrynic acid. Chromatofocusing resolved the GSTs into seven isoenzymes with pI values ranging from 7.96 to 9.6. The primary isoenzymes (pI 8.6) were responsible for more than 94% of the overall activity and consisted of two semi-identical subunits. The study successfully purified and characterized rabbit liver GSTs, revealing their subunit composition, isoelectric points, and substrate specificity. The findings suggest that rabbit liver contains multiple isoenzymes with similar immunological properties, with the primary isoenzymes responsible for most of the enzymatic activity. This purification and characterization offer insights into the enzymatic properties and functional diversity of GSTs in animal tissues.The effect of various inhibitors and the substrate activity of the rabbit liver was tested..

Keywords: Glutathione, Glutathione S-Transferases, Affinity Chromatography, Chromatofocusing, CDNB, and GST.

Introduction:

Glutathione-S-transferases (GSTs, EC 2.5.1.18) are multifunctional enzymes involved in the detoxification of a wide array of xenobiotics and endogenous compounds.

These enzymes play critical roles through both their catalytic and non-catalytic activities, contributing significantly to cellular protection from oxidative stress, inflammation, and tissue damage (Booth et al., 1961; Jakoby, 1978; Habig, 1980).

GSTs catalyze the conjugation of the tripeptide glutathione (GSH) to electrophilic substrates, facilitating their excretion or neutralization (Mannervik & Awasthi, 2006). Moreover, GSTs exhibit various non-catalytic roles, including involvement in signal transduction, apoptosis, and

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cellular transport, underscoring their broad functional significance (Hayes et al., 2005; Sies & Jones, 2020).

The protective role of GSTs against oxidative damage has been well-established, with GSTs acting as a first line of defense against reactive oxygen species (ROS) and other free radicals generated during normal metabolism or in response to environmental toxins (Tew et al., 2011). In particular, GSTs are critical in protecting tissues, especially in the liver, which is exposed to various endogenous and exogenous toxicants (Dai et al., 2020). Over the years, a diverse array of GST isozymes has been isolated from various species, primarily from human, rat, and mouse liver tissues, revealing considerable functional diversity across these forms (Habig & Jakoby, 1988). These isozymes are categorized into different classes based on their substrate specificity, immunological properties, and amino acid sequences (Board et al., 2011).

Recent studies have explored the similarities and differences between the GST isozymes present in the livers of mammals, including the functional variations that exist between species. For instance, Dai et al. (2020) identified notable differences in the GST isoforms between species, with some isozymes exhibiting distinct affinities for specific substrates, such as xenobiotics and endogenous molecules like bilirubin. In the rat liver cytosol, at least 12 electrophoretic forms of GST have been documented, each exhibiting different properties and substrate preferences (Gillham, 1973; Hayes & Chalmers, 1983; Hayes, 1984; Ahmed et al., 2020). This variety suggests that gene duplication and subsequent divergence have played significant roles in the evolution of the GST superfamily, contributing to their functional complexity (Nei & Rooney, 2005).

The interaction of GSTs with bilirubin has garnered particular attention due to its potential involvement in bilirubin metabolism and detoxification. Several studies have demonstrated that GSTs, especially those in the liver, exhibit a strong affinity for bilirubin, and recent investigations suggest that bilirubin binding occurs not only at the catalytic site but also at other distinct regions of the enzyme (Bhargava et al., 1978; Mannervik & Awasthi, 2006). This indicates a broader role for GSTs in the regulation of bilirubin levels and highlights the potential therapeutic implications for diseases associated with bilirubin accumulation, such as jaundice and certain types of liver dysfunction.

In this study, we describe the purification and characterization of various glutathione S-transferase forms from rabbit liver, expanding on the previous work done in other species. We provide evidence of immunological similarities between the electrophoretic forms of the enzyme, furthering our understanding of GST diversity in non-human species. Additionally, the impact of specific chemical inhibitors on GST activity in rabbit liver is investigated, offering insights into the modulation of GST function and its potential therapeutic applications in oxidative stress-related conditions.

Materials and Methods

Materials

Animal and tissue

One year-old white rabbits (*Lepus arabicus*) were obtained from Yarmouk university animal house. Immediately after the animals were killed, fresh liver was removed packed in ice and brought to the laboratory and either processed directly or freezed at -20˚C until use. For antibody production 4-month white rabbits raise and bred at Yarmouk university animal house was used.

Chemicals:

Reduced glutathione(GSH), reduced glutathione-linked agarose, gel filtration protein standard, SDS-electrophoresis protein standards, sodium dodecyl sulphate (SDS), commassie brilliant blue R-250,rtis-base,bovine serum albumin(BSA), complete and incomplete Freuds adjuvants, sodium azide, B-mercaptoethanol, acrylamide, N,N- ethylene bisacrylamide, sephadex G-100 were purchased from sigma chemical company (Mo, USA).1-Chloro-2,4-dinitrobenzene (CDNB). Bromophenol blue was obtained from BDH laboratory reagents (England).

Polybuffer exchange94, and polybuffer74 were ordered from pharmacia fine chemicals (Uppsala, Sweden). Tetramethylethylene-diamine was purchased from Bethesda research laboratories (USA).Barbitol buffer was purchased from Gelman (USA). The herbicide (oxadiazolone) was obtained from supleco Inc.(Switzerland), and the insecticide (diazinone) was obtained from Societ de usine chimiques Rhone poulenc (France).

All other chemicals were obtained from commercial sources and of high purity available.

Methods

Tissue Extract Procedure

A quantity of 20 g of rabbit liver was chopped into small pieces, combined separately with 0.1 M potassium phosphate buffer pH 6.5 (1:2 w/v), and homogenized for one minute at maximum speed in a Waring blender. All subsequent procedures were completed at 0–4˚C, unless otherwise noted. Glass wool was used to filter the supernatant after the homogenate was centrifuged at 37,0000 Xg for 30 minutes using an MSE.Europa24 M centrifuge. The pellet was then disposed of.

Assay of GST

The activity of glutathione S-transferase toward 1,2-chloro-2,4-dinitrobenzene, 2,4-dichloronitrobenzene, p-nitrobenzylchloride, p-nitrobenzoyl azide, ethacrynic acid, 1,2-epoxy-3-(p-nitrophenoxy)propane, and 5,β-androstan-3, 17-dione was measured spectrophotometrically using a PYE Unicam SP 8-400 spectrophotometer.

, nearly in accordance with the description provided by Habig et al. (1974). µmol/min was the measurement of enzyme activity at 25°C, and µmol/min/mg protein was the expression of specific activity. Using the method of Lowry et al. (1951) and bovine serum albumin as the standard, the concentration of proteins was measured.

Purification of Glutathione S-transferase

Swollen reduced glutathione agarose beads were placed in a 1×5cm column after being swollen in 0.1M potassium phosphate buffer pH 6.5. At a flow rate of 30 mLs per hour, the column was equilibrated using the same buffer. A 20 ml per hour flow rate of the 37000 xg. Supernatant (400 mg proteins) was introduced into the column. The protein absorbance at 280 was then completely cleaned off the column using equilibrating buffer until it reached zero again. To get rid of the nonspecifically bound proteins, the column was again washed with 100 mLs of the same buffer that contained 0.2 mL of NaCl. Then, using 50 mM Tris-HCl buffer pH 9.6 with 10

mM reduced glutathione, the enzyme was eluted. The fractions (2 ml each) with the highest enzyme activity were combined and dialyzed against 40 volumes of 0.1M potassium phosphate buffer pH 6.5 for 14 hours straight. Amicon pM 30 membrane was used for ultrafiltration to concentrate the enzyme.

Separation of isoenzymes by chromatofocusing .

Chromatofocusing was carried out in accordance with the directions provided by the manufacturer (pharmacia). After the enzyme from rabbit liver was isolated using affinity chromatography as previously mentioned, it was applied individually to a chromatofocusing column (0.9 × 30 cm) that was filled with poly buffer exchanger-94gel. The enzyme was concentrated to around 2 ml using an Amicon PM 30 membrane. A 0.025M tris-HCl buffer with a pH of 9.4 was used to preequilibrate the column. 270 mLs of pH 4.0 poly buffer (94: H2O) were used to elute the transferase. Each of the two-mL portions underwent an activity assay, and the pH of each fraction was promptly noted. Affinity chromatography was used to separate the poly buffer from the mixture of fractions with the highest enzyme activity.

Determination of Molecular Weight

12% SDS-polyacrylamide gel electrophoresis, as described by Laemmli (1970), was used to estimate the molecular weight of the subunits of the glutathione S-transferase purified from the rabbit liver. The protein standards used in this process were phosphorylase B (94,000), bovine serum albumin (67,000), egg albumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and lactalbumin (14,400).

Antibody Production

Rabbits were used to produce antibodies against pure glutathione S-transferase from their livers, exactly as described by Hunaiti and Kolattukudy (1984). The isolated enzyme (around 350µg) was well combined with the complete adjuvant (1:1v/v) of Freud. The resulting emulsion (0.9ml) was then injected (125µg) at two-week intervals with the incomplete adjuvant (1:1v/v). The rabbit was bled two weeks following the second injection, and the antiserum was extracted using centrifugation at 4000 xg for ten minutes.
By using immunotitration, It was determined how the anti-enzyme from rabbits affected the enzymatic action.200µl of antiserum at varying doses was used to incubate aliquots (20µg) of the enzyme, which were then diluted to 15 µl using rabbit serum as control. Using 1-chloro-2,4-dinitrobenzene as a substrate, the enzymatic activity in the supernatant was measured 10 minutes after 5 hours at 4^{o}C.

Ouchterlony double immunodiffusion

The Ouchterlony double diffusion was performed on microscope slides using 1% agarose on 75mM barbital buffer pH 8.8, containing 0.02% sodium azide. The mixture was boiled and poured onto microscopic slides. Wells of 2 or 4 mm diameter were punched in the gel, and 10ml of crude extract from the rabbit liver was added to the outer wells and 15µl of each antisera to the center well. The slide was allowed to develop for 24-48 hours in a humid atmosphere. The slide was then washed with 0.9% NaCl in distilled water and stained with 0.1% comassie brilliant blue R-250, containing 5% acetic acid.

Inhibition study

Solutions of sodium azide, bromophenol blue and bisacrylamide were prepared in ethanol and used to inhibit the enzyme from the rabbit liver. Two different concentration of each inhibitor (1 and 2mM final concentration) were used 20µg of the enzyme was incubated for 10 min with the inhibitors and 0.1mM reduced glutathione in a total volume of 3ml made up with 0.1M potassium phosphate buffer pH 6.5. After the incubation period, the reaction was started by the addition of 1Mm 1-chloro-2,4-dinitrobenzene and the enzyme activity was measured as described above. Controls without inhibitors were accompanied each assay.

3. 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 4.13µ mole/min/mg.

Purification of glutathione S-transferase

Using glutathione linked agarose, a fast purification 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 (Fig.1).

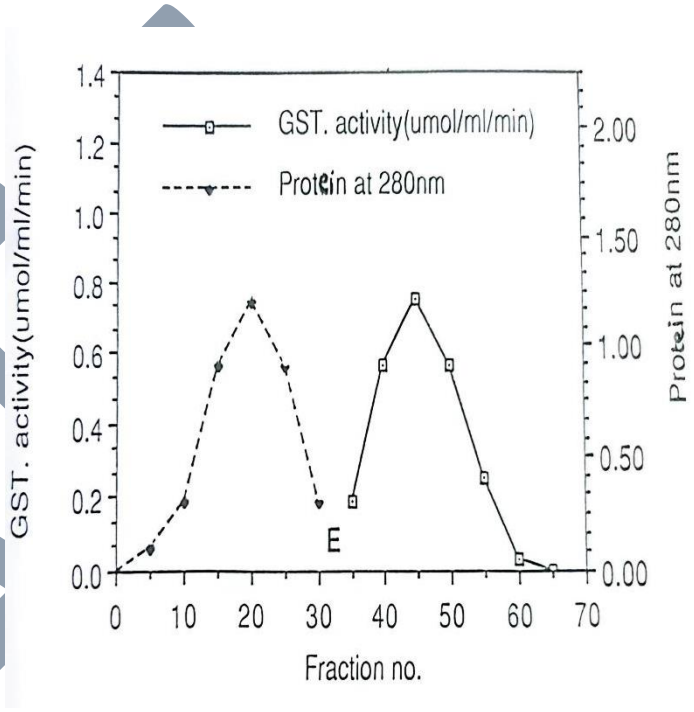

Fig.1: Purification of rabbit liver glutathione S-transferase by glutathione-linked agarose affinity chromatography. Protein was measured at 280nm after 1:40 dilution with0.1M potassium phosphate buffer pH 6.5. E:elution buffer
- Other experimental details are given in the text.

The enzyme was successfully recovered in a single sharp peak by 10 mM reduced glutathione in 50Mm Tris-HCl buffer pH 9.6 (fig.2). This purification procedure resulted in over 78% ecovery

of the applied glutathione S-transferase activity from rabbit liver and about 22 purification fold Table1.

Table 1. Glutathione S-transferase purification from rabbit liver. In the text, experimental details are provided.

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

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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 (Fig.1).

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 (Fig2).

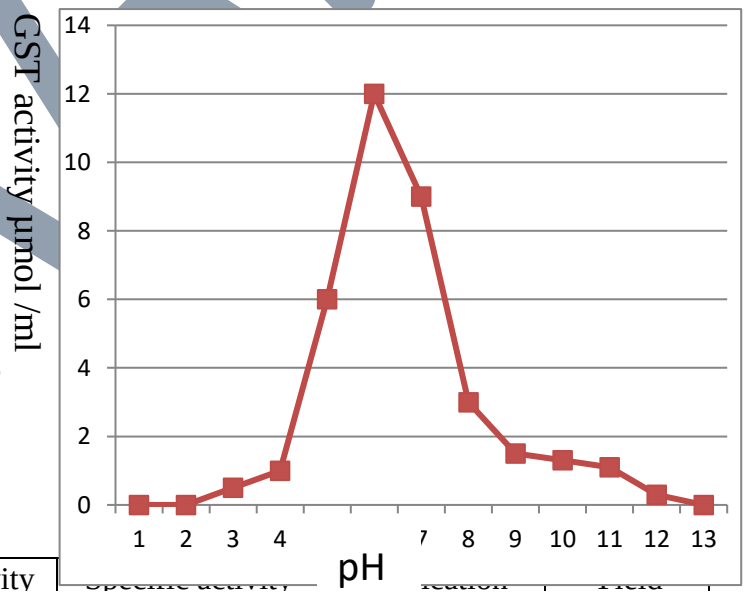

Fig.2 The effects of pH on glutathione S-transferase activity in the liver of rabbit

Fraction	Total protein (mg)	Total activity µmol			
Supernatant	346	1430	4.13	1	100
Affinity chromatography	12.36	1120	90.61	22	78.3

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 (Fig.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 (Fig. 3).

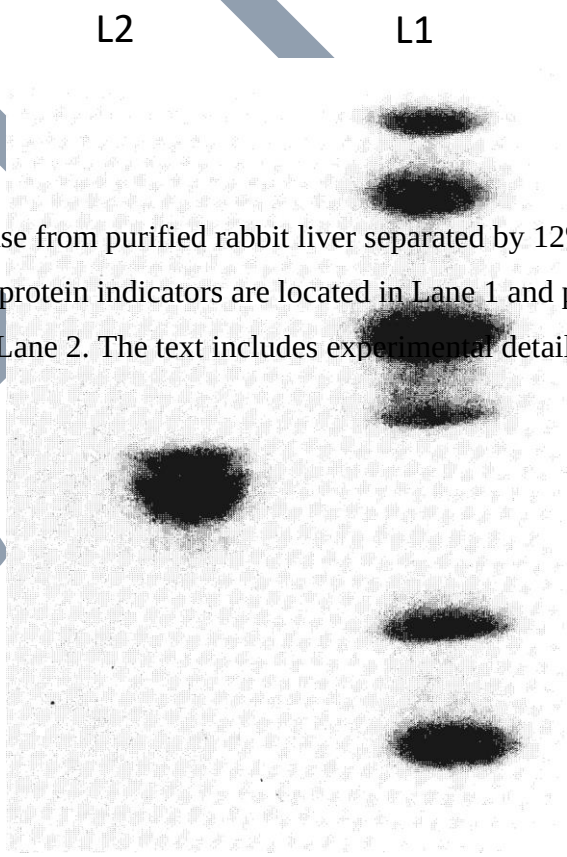

Figure 3: Glutathione S-transferase from purified rabbit liver separated by 12% SDS-gel electrophoresis. Standard protein indicators are located in Lane 1 and pure rabbit liver enzyme is located in Lane 2. The text includes experimental details.

Resolution of the Isoenzymes

Seven peaks of activity were identified in the transferase by means of an isoelectric fractionation using Sephadex polybuffer exchanger 94 (Fig.). At pH of approximately 8.7, the majority of rabbit liver GST isozymes showed strong activity.

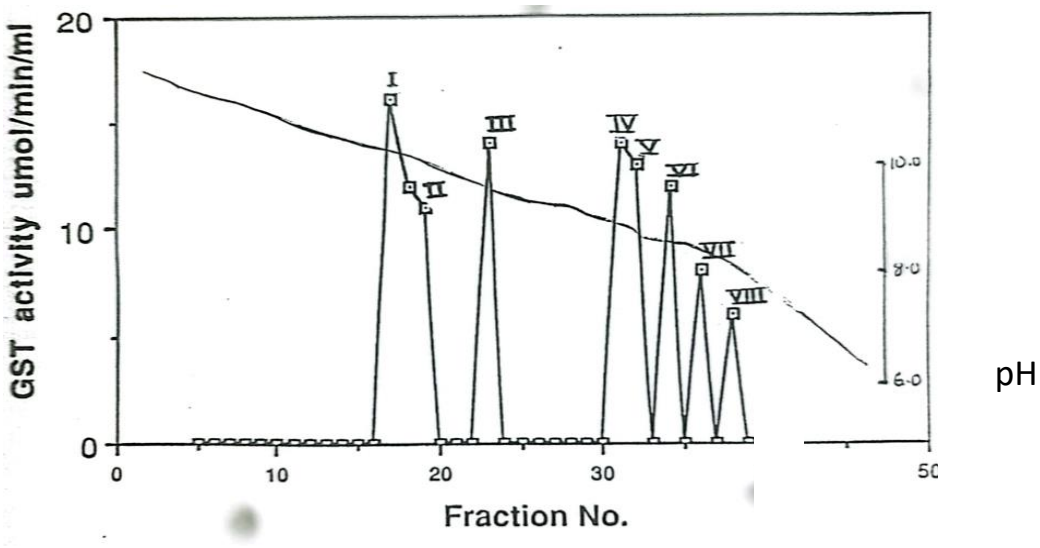

Fig.4 Purification of various isoenzymes of rabbit liver glutathione S-transferase by chromatofocusing Peak1 pI 9.6), Peak II(pI 9.2) , Peak III (pI 8.6) , Peak IV (pI 8.3), (Peak V (pI 8.1), Peak VI (pI 8.05), Peak VII)

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.

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

Enzyme (30~g) was preincubated for 2 minutes at room temperature with inhibitors and 1 mM GSH in 3 ml of 0.l M potassium phosphate buffer pH 6.5. The reactions were then started by adding 1 mM 1-chloro-2,4-dinit robenzene.

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 Oucherlony double-diffusion research

revealed the presence of the rabbit antibody produced against the principal peak (pI 8.7) of the chromatofocusing column.

Inhibitors	(%) of Rabbi
Sodium azide 1 mM	
Sodium azide 2 mM	
Bromophenol blue 1 mM	
Bromophenol blue 2 mM	
Bisacrylamide 1mM	
Bisacrylamide 2mM	

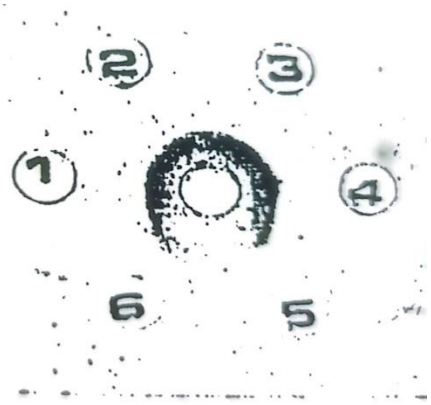

Fig.5 5 Analysis of double diffusion in Oucherlony. Rabbit antiserum produced against the primary isoenzyme (pI 8.7) was present in the center well in amounts of 20µ1, whereas the purified isoenzyme was found in increasing amounts in the outside wells 1, 2, 3, 4, 5, and 6.

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

The antigen, which can be an unprocessed or pure enzyme, generated a single precipitin line (Fig. 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 seven electrophoretic forms show no spurs and completely merged immunoprecipitin lines, suggesting that they are immunologically quite similar.

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 shown inhibition at relatively high doses (Table 3).

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 (Habig WH et al., 1974; Kamisaka K et al., 1975; Huang R, Jiang Z, Zhang H, 2019; Jiang L, Xu Y, Zhang Y, 2021). The GSH-S-transferases identified in rabbits and rat liver (Mosialou E, Tsiftsoglou AS, 1994; Liu H, Chen Z, Wang X, 2021) 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 (Singh et al., 1985; Shi Y,

Wang F, Li Z, 2021; Khan M, Akhtar M, Rehman S, 2020). With subunit molecular weights ranging from 22,500 to 26,000, these enzymes are heterodimers (Reddy et al., 1983; Huang R, Jiang Z, Zhang H, 2019). 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 seven different electrophoretic forms (Feng L, Zhou Z, Liu J, 2021).

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 seven forms of the enzyme (Dao et al., 1984; Singh et al., 1985; Hunaiti AA, Soud MA, 2000). This is in contrast to liver enzymes from rats or mice. If the seven transferases present in rabbit liver exist at all, it is unclear how they relate to one another. All seven may have diverged from a single gene from a common ancestor. In that instance, the antigenic characteristic was clearly preserved. Reddy et al. (1983) 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 (Fig. 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 is an effective inhibitor of glutathione S-transferase. Because of this, BPB is an invaluable tool for studies and potential therapeutic uses involving GST inhibition (Guo Q, Wang Y, Sun S, 2020). Even yet, great care was taken to wash the chromatofocusing column with a lot of salt. It is currently unclear how the seven 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

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

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

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