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Characterization of S-thiolation and expression of the [pi] class of rat glutathione S-transferase and its application as a marker for rat hepatocarcinogenesis

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Characterization of S-thiolation and expression of the π class of rat glutathione *S*-transferase and its application as a marker for rat hepatocarcinogenesis

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GENERAL INTRODUCTION

Dissertation Organization

This dissertation contains three papers, each of which has its own abstract, introduction, materials and methods, results, discussion, and references. These papers will be submitted to scientific journals for publication, with modifications to meet the journal's format requirements. Following the last paper is a general conclusions chapter followed by the references cited in the general introduction.

Literature Review

Role of Antioxidant Enzymes in Cellular Defense Systems in Carcinogenesis

Reactive oxygen species, which are reactive radicals (superoxide anion, $O_2^{-\bullet}$, hydroxyl radical, HO•, peroxyl radicals, ROO•) or non-radicals (hydrogen peroxide, H_2O_2; singlet oxygen, O_2^*), and radicals of carbon, nitrogen and sulfur are implicated in both initiation and promotion in multistage carcinogenesis (Sun 1990, Cerutti 1985). During this process, DNA damage such as chain breaks and base modifications, lipid peroxidation in cellular membranes (Gardner 1989), enzyme alterations such as protein fragmentation in plasma (Wolff et al. 1986) and protein

oxidation containing reactive sulfhydryls (Miller 1990), and cell death occur.

The cellular free radical scavenger system is composed of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), and nonenzymatic antioxidants such as sulfhydryl compounds, NADPH, vitamins C and E, and β-carotene. This system is thought to function as an inhibitor during both the initiation and promotion of carcinogenesis (Sun 1990).

Antioxidant enzymes may be one component of the defense systems to protect the body against oxidative stress (Boutwell 1964). The activities and concentrations of several antioxidant enzymes are suppressed during the development of neoplasia. Activities of superoxide dismutase (SOD) and catalase (CAT) in nitrosoguanidine and SV-40 virus-transformed mouse liver cell lines are lower than in the normal cell lines (Sun et al. 1989). These transformed cell lines have lower catalase mRNA level through down-regulation of catalase gene expression and less catalase protein than in nontransformed normal mouse liver cell lines (Sun et al. 1993). Total glutathione peroxidase (GPX) activities in tumor tissue from humans such as lung, colon

and stomach are decreased compared with the activities in the normal tissues (Howie et al. 1990).

Oxidative Stress and Carcinogenesis

Superoxide anion ($O_2\bullet^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\bullet OH$), which are formed from the oxygen molecules by sequential one electron reductions, the hydroperoxy radical, $HO_2\bullet$ (protonated form of $O_2\bullet^-$), singlet molecular oxygen, O_2^{1*}, alkoxy radical, $RO\bullet$, peroxyl radical, $ROO\bullet$, organic peroxide, ROOH and excited carbonyl, ^3RO are the major forms of reactive oxygen species (ROS) (Willson 1985). These ROS show mutagenicity in ΦX174DNA (Lobe et al 1988), *Salmonella typhimurium* (Moody et al 1982), and mammalian cells (Cunningham et al 1984). DNA damage, such as base damage, single/double-strand DNA breaks and chromosomal abnormalities by ROS may induce molecular damage that cause mutagenicity and carcinogenecity (Cerutti 1985). The xanthine/xanthine oxidase system induces chromosomal aberrations in Chinese hamster cells (Sofuni 1984) and in V79 cells (Iwata 1984). $O_2\bullet^-$ produced extracellularly by human neutrophils promotes and transforms cultured mouse embryo fibroblast cells (C3H/10T1/2) (Weizman 1985). Tumor promoters such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)

show promoting activity through an oxygen mediated mechanism (Kensler and Trush 1984) and ROS are known to be important components of the tumor promotion process (Trush and Kensler 1991). These facts strongly suggest that ROS may be involved in carcinogenesis, and that the antioxidant defense systems may play important roles in cancer development in which oxidative stress is involved.

Protein S-Thiolation/Dethiolation during Oxidative Stress

A disturbance of the reduction/oxidation balance in cells in favor of the latter can be termed oxidative stress (Sies 1985). The concentration of proteins that possess reactive sulfhydryls (-SH) may be more than that of GSH (Okura et al. 1988, Hu et al. 1988). One of the early cellular modifications of proteins during oxidative stress is protein *S*-thiolation. S-thiolation is the formation of mixed disulfides between protein sulfhydryls and low molecular weight thiols, such as glutathione (S-glutathiolation) and cysteine (S-cysteylation). Dethiolation is reduction of the mixed disulfides to sulfhydryls (Chai et al. 1994a,b, Schuppe-Koistiinen et al. 1994).

It has been proposed that there are two mechanisms of protein S-thiolation/dethiolation (Thomas 1995). The first

mechanism of protein S-thiolation may occur by thiol/disulfide exchange after oxidation of cellular glutathione to glutathione disulfide. The second mechanism of protein S-thiolation may occur by reaction of reduced gltathione (GSH) with protein thiyl radicals (protein-$S^{\bullet}$) formed by reactions initiated by several different species.

A direct relationship between tissue glutathione disulfide and the amount of S-glutathiolated protein suggests that thiol/disulfide exchange is a major mechanism for protein S-thiolation (Bellomo et al. 1987, Brigelius et al 1982). In potential reactions (Thomas 1995) for thiol/disulfide exchange, the anionic form of the protein sulfhydryl (protein-S^{-}) reacts with low molecular weight disulfides (RSSR) such as glutathione disulfide to form the protein mixed disulfides (protein -S-SR). The role of protein S-thiolation/dethiolation by this mechanism has been well studied in glucose metabolism. The S-thiolation of several enzymes stimulates gluconeogenesis, the hexose monophosphate shunt and glycogenolysis, and inhibits glycolysis and glycogen synthesis resulting in increasing NADPH production. The enzymes activated by mixed disulfide formation are glycogen phosphorylase a (active form), and fructose-1,6-bisphosphatase, whereas the enzymes inhibited by

mixed disulfide formation are glycogen synthase I/D, phosphofructokinase, hexokinase, pyruvate kinase, and glycogen phosphorylase phosphatase (Brigelius 1985). This metabolic regulation by S-thiolation/dethiolation can provide secondary antioxidants by generating NADPH (Schuppe-Koistinen et al. 1994, Ravichandran et al. 1994, Gilbert et al. 1984).

In the second mechanism for protein S-thiolation/dethiolation, protein thiyl radicals produced by reactions (1) and (2) can lead to protein *S*-thiolation through reaction (3) and (4).

(1) $\text{protein-S}^- + \text{ROO}^\bullet \rightarrow \text{protein-S}^\bullet + \text{ROO}^-$

(2) $\text{protein-SH} + \text{R}^\bullet/^\bullet\text{OH} \rightarrow \text{protein-S}^\bullet + \text{RH}/\text{H}_2\text{O}$

(3) $\text{protein-S}^\bullet + \text{G-SH} \rightarrow \text{protein-S-S}^{\bullet-}\text{-G} + \text{H}^+$

(4) $\text{protein-S-S}^{\bullet-}\text{-G} + \text{O}_2 \rightarrow \text{protein-S-S-G} + \text{O}_2^{\bullet-}$

The S-thiolated proteins may be reduced to their thiol form by protein dethiolation which may occur either non-enzymatically by thiol/disulfide exchange with a reduced thiol such as glutathione or enzymatically by "dithiol" proteins such as glutaredoxin (also called thioltransferase), or thioredoxin, (Thomas & Sie 1992). Glutaredoxin can be reduced by reduced glutathione (Miller et al 1991), thioredoxin can be reduced by thioredoxin reductase in the

presence of NADPH, and protein disulfide isomerase can be reduced either by NADPH or by glutathione (Thomas & Sies 1992).

Protein S-thiolation produced by a direct reaction of radical species with proteins can prevent the reaction of the reactive radical species with other molecules (Willson 1985). Dethiolation then reduces the protein to its original state, thus contributing to the detoxification of free radical generating species (Thomas & Sies 1992) (Figure 1). Although diverse model systems and intact cells remain to be investigated, the evidence that reactive oxygen species produced by the xanthine oxidase reaction with xanthine and reduced glutathione initiate S-thiolation of purified phosphorylase b, and creatine kinase (Miller et al 1990, Park & Thomas 1988). Also, the facts that the release of O_2^- from human neutrophil induced by phorbol-12-myristate-13-acetate (PMA) involves S-thiolation of various proteins including actin (Shen et al. 1993), and that these S-thiolated proteins return to their original forms by dethiolation or reduction process strongly suggest the protective role of S-thiolation/dethiolation in oxidative stress. Therefore, protein S-thiolation/dethiolation may also protect cells

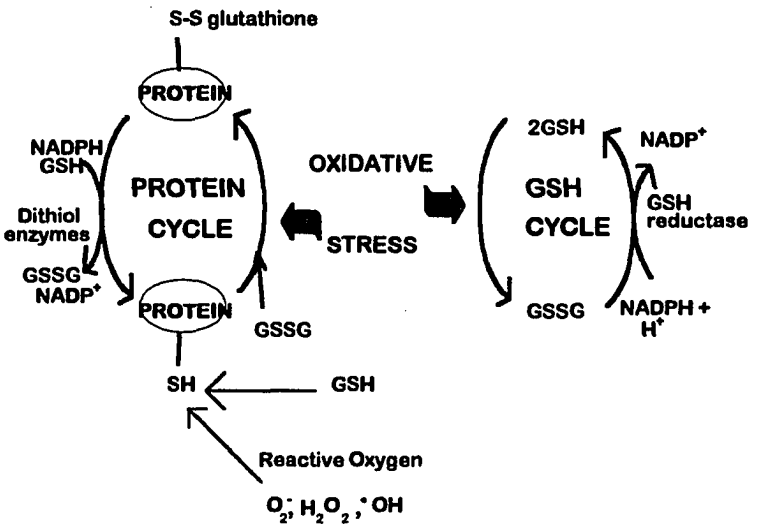

Figure 1. Protein S-thiolation/dethiolation and the glutathione cycle.

during the oxidative stress associated with carcinogenesis (Figure 1).

Glutathione *S*-Transferase Classification and Their Function

The superfamily of glutathione *S*-transferases (EC 2.5.1.18) are phase II enzymes, which conjugate glutathione with a variety of reactive electrophiles by catalyzing the nucleophilic attack of the sulfur atom of glutathione on

electrophilic groups in a second substrate (Mannervik & Danielson 1988). According to substrate specificities, sensitivities to certain inhibitors, cross-reactivities with specific antibodies, isoelectric point, and N-terminal amino acid sequences, glutathione *S*-transferases may be grouped into at least four species-independent gene classes, termed alpha, mu, pi, theta of cytosolic enzymes, and a class of microsomal enzymes (Tsuchida & Sato 1992). Taylor *et al.* (1993) hypothesize an evolutionary relationship of glutathione *S*-transferase classes based on cDNAs encoding these enzymes, in which pi/alpha/mu class precursor occurred from a theta-gene duplication, class mu separated from the common alpha/mu/pi precursor, and subsequently alpha and pi diverged. A recent nomenclature system for human glutathione *S*-transferases, which can also be applied to other mammalian species, has been proposed (Mannervik et al. 1992). This new nomenclature system for glutathione *S*-transferases (GST), uses for example, acronyms hGST A1-1, rGST M1-1, pGST P1-1 etc., in which the prefix indicate the species (h, human; r, rat; m, mouse; p, porcine; b, bovine; rb, rabbit; c, chicken; gp, guinea pig); A, M and P indicating gene class alpha, mu and pi; 1-1 indicating a dimer of two type-1 subunits (Dirr et al. 1994). The

cytosolic glutathione *S*-transferases are dimeric proteins which can be assembled noncovalently as homo- and heterodimeric combinations of different subunits, about 22-26.5 kDa each, from the same gene class (Mannervik & Danielson 1988). Class pi and class theta seem to be produced by their single gene resulting in a single homodimeric isoenzyme form, with class alpha and mu classes are produced by multiple genes and intraclass subunit hybridization resulting in several homo- and hetero-dimeric isoenzyme forms (Dirr et al. 1994).

The glutathione *S*-transferases are multifunctional proteins of which the first role of the enzymes is to catalyze the detoxification of a large number of hydrophobic electrophiles, including quinones, e.g. Benzo(a)pyrene 1,6- and 3,6-quinones produced by microsomal oxidation of benzo(a)pyrene; lipid and nucleic acid hydroperoxides, e.g. arachidonate hydroperoxide (associating a selenium-independent peroxidase activity with GST) epoxide products of oxidation of C=C double bonds *in vivo* e.g. cholesterol α-epoxide; alkenes, e.g. α,β-unsaturated carbonyl compounds such as 4-hydroxyalkenals produced by lipid peroxidation (Mannervik & Danielson 1988). Besides their catalytic activities or conjugation reaction for detoxification,

glutathione *S*-transferases are involved in leukotriene (e.g. GST M6-6) (Tsuchida et al. 1987), prostaglandin (GST A1-A1) (Ujihara et al. 1988), and hepoxilin (GST M4-4) (Laneuville et al 1990) metabolism. Ligandin (GST A1-1 and A1-2) and other forms, including GSTs M3-3 and M4-4, have activity to bind steroid and thyroid hormones (Homma et al. 1986, Ishigaki 1986).

The π Class of Glutathione *S*-transferases as Markers for Altered Hepatic Foci Among numerous markers for AHF, Satoh *et al.*(1991) confirmed that a form of glutathione *S*-transferase, which was first purified from the rat placenta and named the placental form, GST-P, by Sato and coworkers (1992),is an exceptionally useful immunohistochemical marker for altered hepatic foci (AHF) in rat hepatocarcinogenesis. Also the π class of human glutathione *S*-transferase can be a marker for certain types of tumors from esophagus, colon, uterine cervix, breast, lung (non-small cell carcinoma), brain (glioma) and skin (melanoma)(Sato 1989, Tsuchida 1989).

As a marker, rGST P1-1 has been used to quantify initiation and promotion of chemical hepatocarcinogenesis, and has advantages as compared with other enzyme markers such as γ-glutamyl transferase (GGT) in which rGST P1-1 is a

consistent response to all foci and nodules in hepatic (pre)neoplastic lesions (Tatematsu et al. 1988), and may specifically identify more rapidly growing preneoplastic lesions (Hendrich et al. 1987). Thus use of rGST P1-1, in preference to other enzyme markers, will provide reliable information and a tool to quantify effects of many carcinogens and/or anticarcinogenic agents during rat chemical hepatocarcinogenesis.

Structure of GST P1-1 The three-dimensional X-ray crystal structure of the class pi glutathione *S*-transferases from pig lung (pGST P1-1) in complex with glutathione sulfonate (GSO_3^-)(Dirr et al. 1994, Dirr et al. 1991, Reinemer, 1991) and from human placenta (hGST P1-1) in complex with S-hexylglutathione (Reinemer et al 1992) has recently been reported. These dimeric proteins are globular molecules with dimensions of 55Å x 52Å x 45Å for pGST P1-1 and 60Å x 58Å x 44Å for hGST P1-1. The amino acid residues counting from NH_2 terminus of pGST P1-1 suunit are composed of 207 amino acid residues, and pGST P1-1 lacks two amino acid residues, glutamine-40 and glycine-41 of hGST P1-1, mGST P1-1 or rGST P1-1, of which the subunit has 209 amino acid residues. Therefore, Cys 14, Cys45, Cys 99, and Cys 167

residues of pGST P1-1 correspond to Cys 14, Cys47, Cys 101, and Cys 169 of hGST P1-1, rGST P1-1, and mGST P1-1.

A subunit from pGST P1-1 is folded into two topologically different domains of different structure: domain I, N-terminal domain (residues 1-74, 29Å x 24Å x 23Å) which is a structure of α-helices/β-sheet with the folding topology βαβαββα, and domain II, C-terminal domain (residue 81-207, 42Å x 26Å x 23Å) which is an all α-structure. These two domains are connected by linker residues 75-80 (Figure 2) (Dirr et al. 1991).

The π class of glutathione *S*-transferases was proposed to have two distinct functional regions: a hydrophilic G-site for binding substrate glutathione, and an adjacent hydrophobic H-site for binding structurally diverse electrophilic substrates in its active site (Mannervik & Danielson 1988). Residues providing the environment for the G-site are highly conserved within the gene class making glutathione *S*-transferases highly specific towards reduced glutathione as the thiol substrate (Dirr et al. 1994). Crystallography of pGST P1-1 with glutathione sulfonate (GSO_3^-), a competitive inhibitor, recently has identified the glutathione binding site of the enzyme (G-site) which is

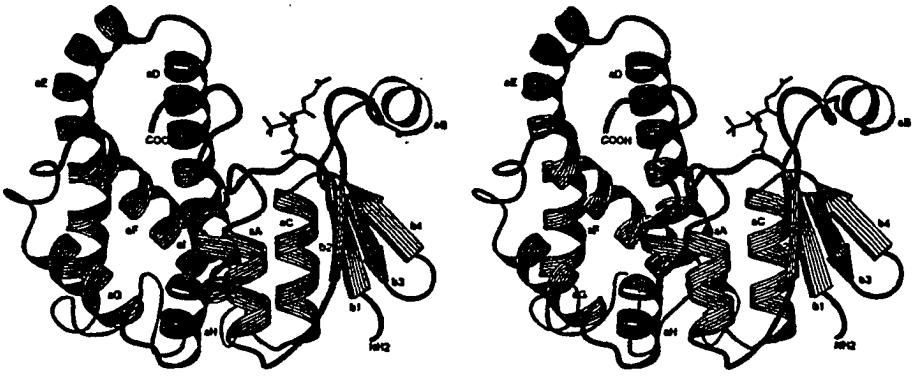

Figure 2. Stereo-ribbon diagram of a subunit of the π group of pig glutathione *s*-transferase with the ligand glutathione sulfonate included (Reiner et al. 1993).

located on domain I, and GSO_3^- binds to a site situated in a cleft between intrasubunit domains (Reinemer et al. 1993). In the binding of glutathione or its analog and pGST P1-1, there are polar interactions between nearly all the polar atoms of the tripeptide and the G-site in domain I of one subunit and one amino acid residue in domain II of the other subunit in the dimer. As seen in Figure 3, on the γ-

glutamyl side of GSH, the α-amino group of glutamyl residue of GSH binds to the Gln62/Asp96 residues, and the α-carboxylate group of glutamyl residue of GSH binds to the Arg 13/Ser 63 and amide group of Ser 63 through salt links. The amide nitrogen (N-H) and carbonyl carbon (C=O) of GSH bind to the Glu 49 residue and carbonyl group of Leu 50 respectively, through hydrogen bond. The C=O of the peptide link between the cysteinyl and glycyl residues of GSH binds to the amide group of Leu 50 by a hydrogen-bond. A salt link is formed with the α-carboxylate of the glycyl residue and Trp 38/Lsy 42/Gln 49 residues of the proteins. The hydrogen bond interaction (Tyr-O-H • $^{-}$S-G) is between the thiol group of glutathione and the hydroxyl group of an invariant Tyr 7. A protonated tyrosine seems to be required to stabilize the GSH thiolated anion at neutral pH by 2.7 kcal/mol for catalytic activity of the enzyme. Although the H-site structures in the crystal structure of glutathione *S*-transferases, identified with substrate-product analogs such as *S*-hexylglutathione, differ somewhat between gene classes, a nonpolar amino acid side chain environment provides the highly hydrophobic protein surface. The H-sites include the active-site loop connecting β1 to α1, the C-terminal region

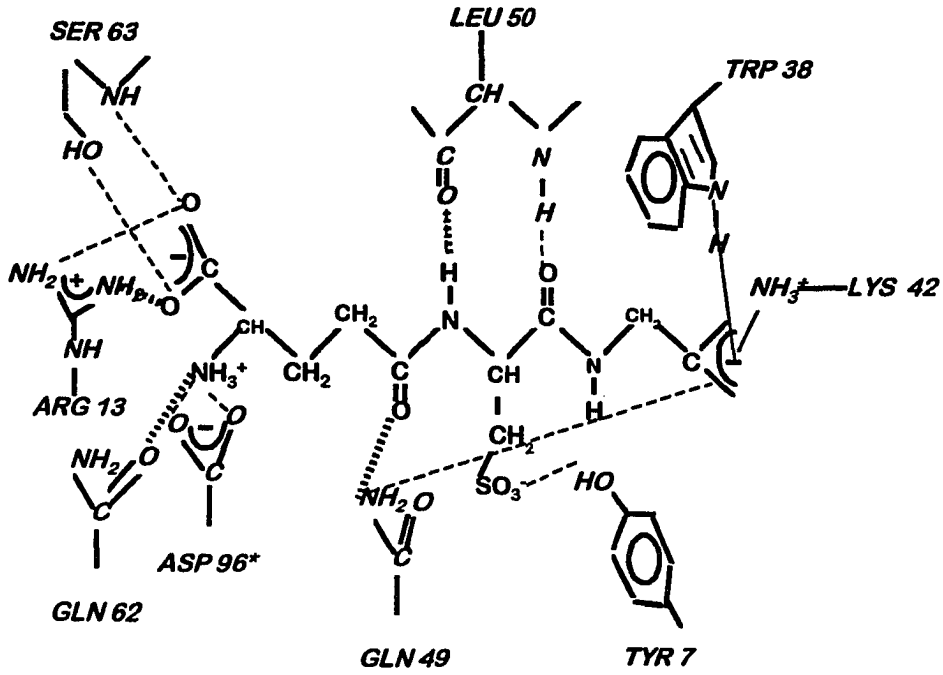

Figure 3. Schematic representation of the interactions in the π class of pig glutathione *S*-transferase (pGST P1-1) with the inhibitor glutathione sulfonate (GSO_3^-)

Asp 96* indicates the residue that is in domain II from the other subunit. Structural components of pGST P1-1 was distiguished by Italic characters.

of α4, and the C-terminal segment of the polypeptide chain which comprises both domain I and domain II of the same subunit.

Very strict recognition at the subunit interface allows class-specific dimerization only from the same gene class (Dirr et al. 1994). Dimeric pGST P1-1 (55Å x 52Å x 45Å) is a globular protein, and the moderate (13%) coverage of accessible surface area upon dimerization can explain the existence of a large cavity formed between the two subunits (Dirr et al. 1991). The intersubunit contact area of pGST P1-1 is mainly composed of hydrophobic residues in a β-turn (residues 45-48), strand β4 and helix α3 in domain I of one subunit and the antipararallel helix pair, α4 and α5, in domain II of the other subunit.

Selenium-independent Glutathione Peroxidase Activity of GST P1-1 The π class of glutathione *S*-transferases not only detoxifies alkylating agents by GSH conjugation, but also possesses selenium-independent glutathione peroxidase activity toward lipid peroxides produced by reactive oxygen species (Kitahara et al. 1983, Meyer et al. 1985).

Reactive Sulfhydryls of GST P1-1 The π class of glutathione *S*-transferases (GST P1-1) has four cysteine

residues per subunit which are Cys 14, 47, 101, and 169 from the N-terminal in GST P1-1 of human, rat, cow and mouse, and Cys 14, 45, 99, 167 in that of pig (Dirr et al 1994). The identification of the reactive sulfhydryls on the subunit of GST P1-1 using selective modifiers of thiol groups of the protein, followed by endopeptidase cleavage, HPLC separation and amino acid sequence analysis shows different results. Cys 47 of GST P1-1 subunit is selectively labeled when N-maleimide derivatives are used for sulfhydryl modification (Bello et al. 1990, Tamai et al. 1990), whereas Cys 47 and Cys 101 are two highly reactive thiols when 7-fluoro-4-sulfamoyl-2,1,3-benzodiazole (ABD-F) is used (Nishihara et al. 1992). Because of less selective reactions in which lysine and histidine can also react with the N-maleimide derivatives, Nishihira and coworkers (1992) suggested that the chemical reactivities or stabilities of the adducts of sulfhydryl modifiers and cysteine residues may account for the difference in the number of reactive cysteines of GST P1-1.

S-thiolation of GST P1-1 and CA III Although none of the four cysteine residues in rGSTs P1-1 are critical for its GST activity (Tamai et al. 1991, Kong et al. 1991), chemical modification of the reactive sulfhydryls (Cys 47 and Cys 101)

in rGST P1-1 by thiol/mixed disulfide exchange with low molecular disulfides such as cystine (Terada et al 1993, Nishihara et al. 1991), by H_2O_2 treatment,and by thiol group modifiers (Nishihara et al. 1992) can markedly inactivate the enzyme activity. This evidence may indicate that S-thiolation/dethiolation of GSTs P1-1 *in vivo* may function to modulate its catalytic activity.

Carbonic anhydrase III (CA III) is a well-studied protein containing reactive sulfhydryls. It has been identified as a major S-thiolated protein in rat hepatocytes (Shen et al. 1993). In addition to S-thiolation/dethiolation of CA III, the fact that CA III is known to be a major soluble protein (5-7% of the soluble proteins) in male rat liver and skeletal muscle (Carter et al. 1981, Geers et al. 1992), yet it has 1-2% the activity of CA II, CA isoenzyme (Tashian 1989, Spicer et al. 1990), and that this protein has two reactive sulfhydryls which are not involved in enzyme activity (Thomas & Sies 1992) suggest that this protein may play a role as an antioxidant activity unrelated to enzyme activity.

Both enzymes may have antioxidant function through protein S-thiolation/dethiolation and the presence of protein thiols of these protein may protect against oxidative stress.

Models of Carcinogenesis

Beginning with studies in mouse skin (Boutwell 1964, Slaga 1982) or rat liver (Pitot et al. 1978, Peraino et al. 1981) as model systems, carcinogenesis in animals is now considered to consist of at least three different and distinct stages: initiation, promotion, and progression (Scherer 1984, Pitot et al. 1991, Pitot 1990, Dragan et al. 1993). Due to difficulty in inducing epidermal tumors by dietary or parenteral administration of putative carcinogens, rat hepatocarcinogenesis has been one of the most extensively studied and most easily quantified experimental multistage carcinogenesis models (Pitot 1990). The stage of initiation results in irreversible genetic alteration of individual hepatocytes (Pitot 1991). These alterations begin to be expressed by clonal proliferation resulting in altered hepatic foci (AHF) or focal lesions at the stage of promotion or preneoplasia, which is at least partly reversible upon removal of the promoting stimulus (Hendrich et al 1987). Finally, enhanced proto-oncogene expression, and malignant neoplasia, e.g., hepatocellular carcinoma, develop from some AHF or preneoplastic cells during the stage of progression (Pitot 1991). Altered hepatic foci (AHF) or "islands" of altered cells become evident after treatment of rats with

hepatocarcinogens. The lesions within a single liver display a high degree of phenotypic heterogeneity. The expression of a wide variety of genes in AHF as monitored by histochemistry, and immunohistochemistry provide a way to analyze the specific quantitative parameters of these lesions in studing their natural history and the development of malignant neoplasia in the liver (Pitot 1991).

Model of Phenobarbital and Fumonisins As Promoters In Hepatocarcinogenesis

Phenobarbital Phenobarbital (PB), has been used as a tumor promoter in the hepatocarcinogenesis model (Peraino et al. 1981, Pitot et al. 1978). Phenobarbital (PB) is able to induce the drug metabolizing microsomal enzyme systems of the liver. Phenobarbital treatment causes oxidative damage to liver by accumulating hepatic lipid peroxidation (Hahn et al. 1976) and induces xenobiotic-metabolizing enzymes, including cytochrome P-450s (Hutton et al. 1979). Induction of cytochrome P-450s by PB may cause the formation of reactive oxygen species, which is thought to contribute to PB promotion (Cerutti 1985, Kensler 1984).

Fumonisins As another promoter, fumonisins were used in the current as a model in hepatocarcinogenesis. Fumonisins are a group of mycotoxins produced by *Fusarium*

moniliforme and *F. proliferatum* (Norred et al. 1991, Gelderblom et al. 1988, Bezuidenhout et al. 1988). Fumonisin B_1 (FB_1) is a common contaminant of corn, often found in amounts of 0.2-2 ppm in corn-based human foods (Sydenham et al. 1991), causing hepatocarcinogenesis in rats fed 50 mg FB_1 /kg diet over 2 years (Gelderblom et al 1991). 50 mg FB_1 /kg diet over 6 months or 1 g FB_1 /kg diet over 4 weeks show tumor promoting activity inducing placental glutathione *S*-transferase (rat GST P1-1)-positive foci (Lebepe-Mazur et al. 1994) or γ-glutamyl transferase (GGT)-positive foci in rat liver (Gelderblom et al 1991). In addition, FB_1 has cancer initiating potential in rat liver, depending on the duration of exposure, for which the effective minimum dose level for initiation of rat hepatocarcinogenesis is within the ranges of 5.4 - 11.6 mg FB_1/100 g body weight per day for 14 days. This suggests that FB_1 is a slow initiating compound (Gelderblom et al. 1994).

Fumonisin B_1 and FB_2 have structural similarities to sphingosine, which led to the hypothesis that fumonisin may interfere in pathways of sphingosine metabolism (Figure 4). Fumonisin B_1 is a potent inhibitor of sphingosine- and

sphinganine-N-acetyltransferase resulting in the inhibition of ceramide biosynthesis (Norred et al. 1992, Schroeder et al 1994), which may lead to deregulation of protein kinase C and proliferation of initiated cells (Norred et al. 1992), perhaps partly by enhancement of oxidative stress in these cells.

Model of Isoflavones As Anticarcinogenic Compounds In Hepatocarcinogenesis

Isoflavones have antioxidant activity (Pratt et al. 1981, Naim et al. 1976, Murata & Ikehata 1964) which may play a critical role in the anticarcinogenic effects of these compounds. The isoflavones, genistin and daidzin, and their aglycones, genistein and daidzein, constitute 90-95% of the isoflavone content of soybeans (Murphy 1982). These compounds have been extensively studied since epidemiological data showed that the lower incidence of breast cancer in Asian women compared with Western women may be attributed to dietary patterns that include soy foods (Dunn, Jr. 1975, Kromhourt et al. 1989, Anonymous 1983). Rats consuming a soy-based diet develop fewer mammary tumors following administration of the carcinogens N-methylnitroso-urea or 7,12-dimethylbenz[a]-anthracene than rats fed isonitrogenous and isocaloric diets without soy (Barnes et al. 1990). In a

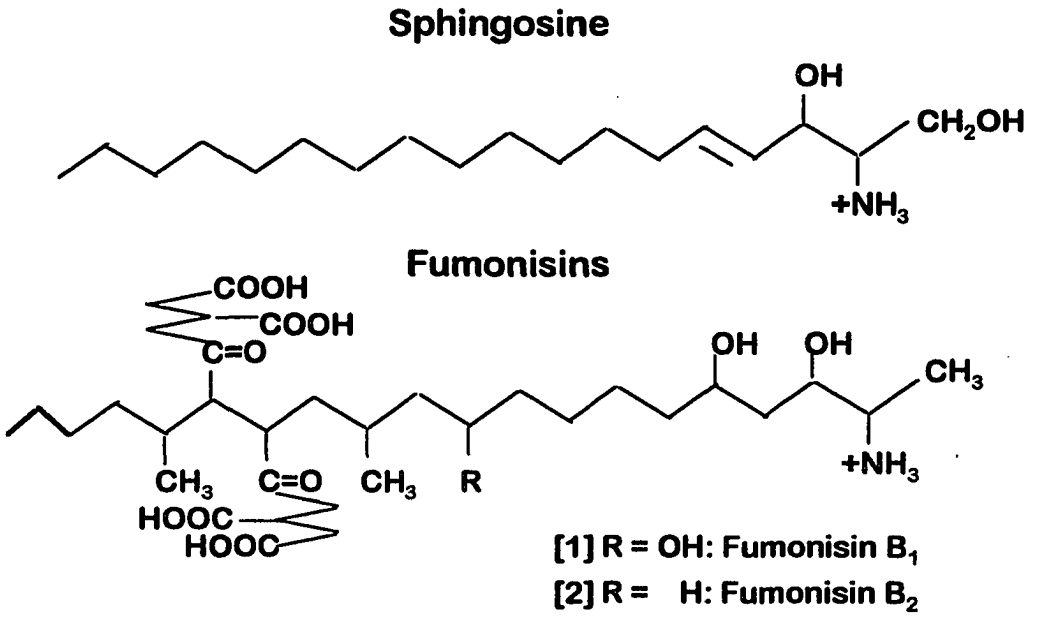

Figure 4. Structure of sphingosine and fumonisins exhibiting the similar long-chain carbon backbone of the molecules.

case-control study of diet and breast cancer in Singapore Chinese, Lee *et al.* (1991) found that soy protein was associated with reduced breast cancer risk in premenopausal women ($p < 0.02$), and suggested that the isoflavones in soy may be responsible. The anticarcinogenic effect may come from biological activities of soybean isoflavones present in soybeans in amounts of approximately 1-3 mg/g (Wang & Murphy 1994).

Isoflavones are phenolic compounds from plant sources sometimes referred to as phytoestrogens, and have similar structure with flavones, in which the B ring is attached to the 3 position of diphenolic ring in isoflavones (Figure 5).

The antioxidant activity of isoflavones may be involved in their anticarcinogenic effects. Reactive oxygen species (ROS) and their subsequent modification of macromolecules, such as protein, RNA, and DNA are implicated in development of multi-stage carcinogenesis, particularly in tumor promotion (Cerutti 1985, Kensler & Trush 1984, Troll & Wiesner 1985). Genistein in the range of 1-150 μM suppresses H_2O_2 formation by 12-O-tetra-decanoylphorbol-13-acetate (TPA)-activated human polymorphonuclear leukocytes (PMNs) and HL-60 leukemia cell line, and also suppresses superoxide anion formation by HL 60 cells (Wei et al. 1993).

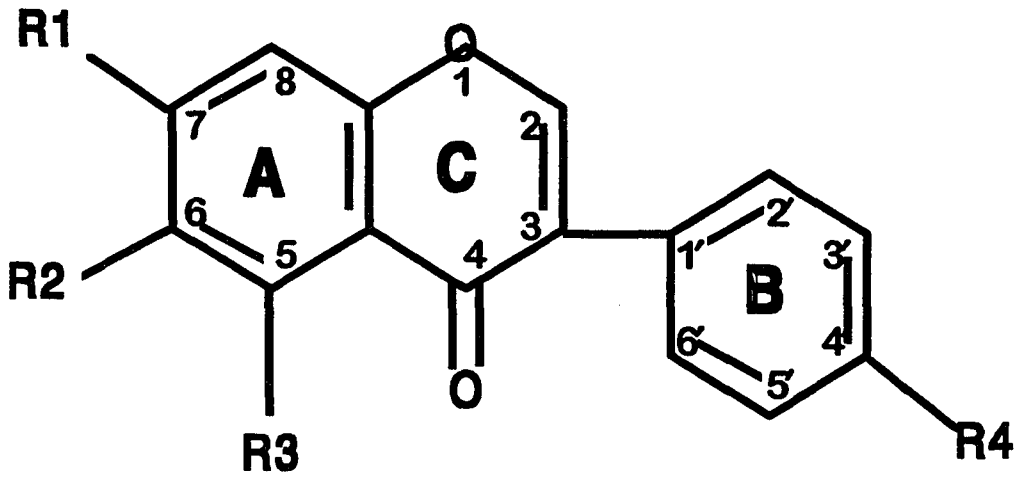

Figure 5. Structure of isoflavone compounds

The structural formulars of major isoflavones are the following;

Genistein, 4',5,7-trihydroxy-isoflavone, R1 = OH; R2 = H; R3 = OH; R4 = OH

Genistin, R1 = *O*-glucosyl; R2 = H; R3 = OH; R4 = OH

Daidzein, 4',7-dihydroxy-isoflavone, R1 = OH; R2 = H; R3 = H; R4 = OH

Daidzin, R1 = *O*-glucosyl; R2 = H; R3 = H; R4 = OH

Equol, a metabolite of daidzein; the same structure of that of daidzein but no ketone group and saturated double bond on 4-position of C ring, R1 = OH; R2 = H; R3 = H; R4 = OH

Glutathione peroxidase (GPX), one of the antioxidant enzymes which protects from O_2-induced damage that increases levels of dangerous peroxides (Pérez-Campo et al. 1993, Verkerk & Jongkind 1992, Lapenna et al. 1992). There are two kinds of cytosolic GPX, (EC 1.11.1.9) 1)Se-dependent peroxidases (sGPX) which uses H_2O_2 and organic hydroperoxides as substrates (Lawrence et al. 1978, Lawrence & Burk 1976), and 2)Se-independent peroxidases which use only organic hydroperoxides such as lipid peroxides and cumene hydroperoxide as substrates (Meyer et al. 1985, Lawrence et al 1978, Kitahara et al. 1983). It hypothesized that enhancement of GPX activity may contribute to antioxidant and anticancer effects of isoflavones (Hendrich et al. 1994).

Isoflavones have other activities as well as their antioxidant effects. Depending on their dose or modes of action, such as competition with estradiol for binding to estrogen receptor, isoflavones can be estrogenic or antiestrogenic.

Isoflavones can be estrogenic, but only at concentrations much higher than that of endogenous estrogen. Based on uterine weight analysis, mice not only consume 1000 fold greater amounts of isoflavones than of estradiol to cause an estrogenic effect, but would have to eat more soy than

possible in a human diet (Farmakalidis et al 1985). *In an vitro* assay using a cell line (Ishikawa-Var I) from endometrial adenocarcinoma , which is sensitive to the stimulatant effect of estrogen on alkaline phosphatase (AlkP) activity, the maximal AlkP activity obtained with the isoflavone compounds tested is as high as that achieved with estradiol. However, the EC_{50} values, the concentrations at which compounds have one-half of their maximum effect on AlkP activity, are quite different among compounds tested, EC_{50}=0.0673 nM for estradiol, 79.8 nM for genistein, 111 nM for equol, and 515 nM for daidzein (Fischer et al 1988).

A single injection of rats with 5 mg of equol, which is formed from daidzein by intestinal bacterial action, (Adlercreutz et al. 1987), shows that increases of uterine weight are observed in this group to the same degree as in the group injected with only 5 μg of estradiol-17β at 24 h. By 36 h after the injection, the rats injected with equol have almost the same uterine weights and the group injected with estradiol-17β has about 1.7-fold weights higher uterine weights as compared with those of the control group (Tang & Adams 1980). A single injection of equol failed to increase uterine cytosol estrogen receptors, despite early higher binding of equol to the nucleus compared with estradiol, and

the uterotrophic effects of multiple injections of equol or of equol plus estradiol are lower than that of estradiol alone in the study.

Daidzein and equol in the range of 10-100 μM compete with 40 nM [^{3}H]estradiol for rat uterine nuclear estrogen type II binding sites (Adlercreutz et al 1992).

The antiestrogenic and antioxidant activities of isoflavones and their inhibitory effects on several enzymes which are involved in cell proliferation such as tyrosine-specific kinases (TPK) (Linassier et al. 1990), casein kinase II (Higashi & Ogawara 1994), DNA topoisomerase (Markovits et al. 1989, Okura 1988), and S6 kinases (Linassier et al. 1990) indicate that isoflavones may contribute to the prevention of many types of cancer.

We are interested in the properties of the π class of rat glutathione *S*-transferase (rGST P1-1) and carbonic anhydrase III (CA III), which may function in antioxidant defense. The function of protein S-thiolation/dethiolation as an antioxidant system may inhibit formation of reactive oxygen species involved in cancer development. In this dissertation, the expression of the π class of rat glutathione *S*-transferase (rGST P1-1) and carbonic

anhydrase III in male F344/N rat liver initiated with diethylnitrosamine (DEN) and promoted with a fumonisin-containing diet is reported in CHAPTER 1. A model system for hepatocarcinogenesis using female F344/N rats initiated by DEN and promoted by phenobarbital and fed with soybean isoflavone extract fed 3 and 11 months was used to investigate the anticarcinogeneic effects of soybean isoflavone extract as a potential anticarcinogen assessed with immunohistochemical staining using rGST P1-1 as a biomarker is reported to show in CHAPTER 2. Finally, the S-thiolation of rGST P1-1 characterized with isoelectric focusing is reported in CHAPTER 3.

CHAPTER 1. EXPRESSION OF THE π CLASS OF RAT GLUTATHIONE *S*-TRANSFERASE (rGST P1-1) AND CARBONIC ANHYDRASE III (CA III) IN MALE F344/N RAT LIVER INITIATED WITH DIETHYLNITROSAMINE AND FED FUMONISIN CONTAINING-DIET

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ABSTRACT The alterations of the π class of rat glutathione *S*-transferase (rGST P1-1) and carbonic anhydrase III (CA III) after initiation by diethylnitrosamine (DEN) and during promotion by fumonisin were investigated in the hepatic cytosolic fraction of male F344/N rats by immunodetection methods using anti-rGST P1-1 and anti-CA III antisera. Diethylnitrosamine (15 mg/kg body weight) was injected intraperitoneally into 20 ten-day old male F344/N rats, and at 4 weeks of age, weaned pups were divided into three groups and fed either the AIN-76 purified diet (40% of kcals from fat), the basal diet containing 69 μmole fumonisin B_1 (FB_1)/kg diet, or the basal diet containing 69 μmole FB_1 reacted with fructose/kg diet.

The rGST P1-1 is a tumor marker of preneoplasia and neoplasia in various rodent and human tissues. Carbonic

anhydrase III is a protein of unknown physiological significance, although it is the most abundant form among CA isoenzymes in rat muscle and male rat liver. During initiation by DEN and promotion of hepatocarcinogenesis by fumonisin treatment, hepatic rGST P1-1 levels was increased by 980% ($p < 0.05$), whereas hepatic CA III level was suppressed by 43% ($p < 0.05$). The reasons for an inverse relationship between expression of rGST P1-1 and CA III during cancer development from male rat liver remain to be clarified. The fact that both rGST P1-1 and CA III have reactive sulfhydryl (-SH) groups, protein -SH groups that are highly reactive with radicals produced during oxidative stress, suggests that rGST P1-1 may replace CA III as an antioxidant protein and that CA III may be a useful biomarker during the development of male rat hepatocarcinogenesis.

INTRODUCTION

Multistage chemical hepatocarcinogenesis, which proceeds through initiation, promotion and progression, has been observed in many animal models (Farber 1984, Pitot & Sirica 1980). We have used diethylnirosamine (DEN) and fumonisin as an initiator and a promoter, respectively to produce male rat hepatocarcinogenesis. Fumonisins, which are a group of mycotoxins produced by *Fusarium moniliforme* and *F.*

proliferatum (Norred et al. 1991, Bezuidenhout et al. 1988), are known tumor promoters (Gelderblom et al. 1988, Lebepe-Mazur et al. 1994). Fumonisins B_1 (FB_1) is a common contaminant of corn, often found in amounts of 0.2-2 ppm in corn-based human foods (Sydenham et al. 1991), causing hepatocarcinogenesis in rats fed 50 mg FB_1 /kg diet over 2 years (Gelderblom et al. 1991). FB_1 is also a tumor promoter inducing γ-glutamyl transferase- and rGST P1-1 positive foci in rat liver (Gelderblom et al. 1988, Lebepe-Mazur et al. 1994). In addition, FB_1 has cancer initiation potential in rat liver depending on the duration of exposure, for which the effective minimum dose level for initiation of rat hepatocarcinogenesis is within the ranges of 5.4 - 11.6 mg FB_1/100 g body weight per day for 14 days, suggesting that FB_1 is a slow initiating compound (Schroeder et al 1994).

The carcinogenic mechanism of FB_1 is not known, but FB_1 is a potent inhibitor of sphingosine- and sphinganine-N-acetyltransferase (Schroeder et al. 1994), and the inhibition of sphingosine biosynthesis by FB_1 may lead to deregulation of protein kinase C and proliferation of initiated cells (Norred et al. 1992). As other tumor promotion, oxidative

stress perhaps enhances proliferation of initiated cells during hepatocarcinogenesis by fumonisin promotion.

DNA damage, such as base damage single/double-strand DNA break and chromosomal abnormality leading to initiation, and tumor promotion by free radical species generating compounds such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) strongly suggest the involvement of reactive oxygen species in multistage carcinogenesis (Sun 1990). Therefore, the antioxidant defense enzymes may play important roles in cancer development in which oxidative stress is involved.

We are interested in the properties of the π class of rat glutathione *S*-transferase (rGST P1-1) and carbonic anhydrase III (CA III), which may function in antioxidant defense. The function of protein S-thiolation (formation of mixed disulfides between protein sulfhydryls and low molecular weight thiols such as GSH) (Grim et al. 1985) and dethiolation (reduction to their original state)(Park & Thomas 1989) was recently proposed by Thomas and Sies (1992) as an antioxidant system which may inhibit formation of reactive oxygen species involved in cancer development.

The GST P1-1 (Tatamatsu et al 1988, Mannervik et al. 1988, Tsuchida et al. 1992) is a good biomarker for rat hepatocarcinogenesis and for some types of human cancer such

as cancers from esophagus, colon, uterine cervix, breast lung (non-small cell carcinoma), brain (glioma) and skin (melanoma) (Sato 1989, Tsuchida 1989). This π class of rat GST P1-1 has advantage to quantify initiation and promotion of chemical hepatocarcinogenesis compared with other enzyme markers such as γ-glutamyl transferase (GGT) because rGST P1-1 may specifically identify more rapidly growing preneoplastic lesions (Hendrich et al. 1987), and to be more consistent response to all foci and nodules in hepatic (pre)neoplastic lesions (Tatematsu et al. 1988). Also the property of this π class enzyme possessing selenium-independent glutathione peroxidase activity toward lipid peroxides produced by reactive oxygen species (Kitahara et al. 1983, Meyer et al. 1985) suggests that GST P1-1 may play a role as an antioxidant enzyme.

Carbonic anhydrase III (CA III) has been identified as a major S-thiolated protein in rat hepatocytes (Chai et al. 1991). The release of $O_2^{\bullet -}$ from human neutrophils induced by phorbol-12-myristate-13-acetate stimulates S-thiolation of CA III (Chai et al 1994a).

Both cytosolic GST P1-1 (Bello et al. 1990, Tamai et al. 1990, Terada 1993) and CA III (Tashian 1989) contain reactive

sulfhydryls. The S-thiolated forms of rGST P1-1 have been identified (Lee 1995). Reactive oxygen species produced by the xanthin oxidase reaction with xanthin and glutathione initiated S-thiolation of CA III (Lii et al. 1994, Chai et al. 1991). Perhaps both rGST P1-1 and CA III have antioxidant function through protein S-thiolation/ dethiolation although this hypothesis needs to be investigated further.

In this study, we have quantified the expression of rGST P1-1 and CA III in livers of male F344/N rats initiated with diethylnitrosamine (DEN, 15 mg/kg body weight) and fed fumonisin-containing diet. Also, a possible method for detoxification of fumonisins by reaction with fructose with heat treatment in which the amino group on fumonisin molecule was reacted with fructose, a reducing sugar by browning or Maillard reaction was investigated.

MATERIALS AND METHODS

Animals and diets

The experimental protocol was reviewed and approved by the Iowa State University Animal Use Committee. Male F344/N rats were obtained from Harlan/Sprague-Dawley (Indianapolis, IN). All animals were allowed access to diets and water *ad*

libitum., and housed in a room maintained at 22°C with lighting 0600 to 1800h.

In the experiment using a fumonisin-containing diet, 20 ten-day old male F344/N rats were injected intraperitoneally with diethylnitrosamine (DEN, 15 mg/kg body weight) (Figure 1). At 4 weeks of age, weaned pups were divided into three groups and fed either the basal diet based on AIN-76 diet but containing 40% of kcals from fat to promote carcinogenesis, the basal diet containing 69 µmole fumonisin B_1 (FB_1)/kg diet, or the basal diet containing 69 µmole fumonisin reacted with fructose /kg diet. FB_1 was purified as described by Lebepe-Mazur *et al.* (1994). Briefly, FB_1 was extracted from *F. proliferatum* strain 5991 corn culture using acetonitrile:water (1:1), and was purified to 47% purity by ethyl acetate partitioning, followed by Amberlite XAD-16- (Sigma Chemical Co. St. Louis, MO), and Lobar C8- (EM Separations, Gibbstown, NJ) reverse phase columns. To prepare the diets containing fumonisin or fumonisin reacted with fructose, 69 µmole FB_1 and 0.1M fructose in 50 mM potassium phosphate buffer, pH 7.0 were reacted over 48 hrs at 80°C, or 69 µmole FB_1 without fructose was treated under

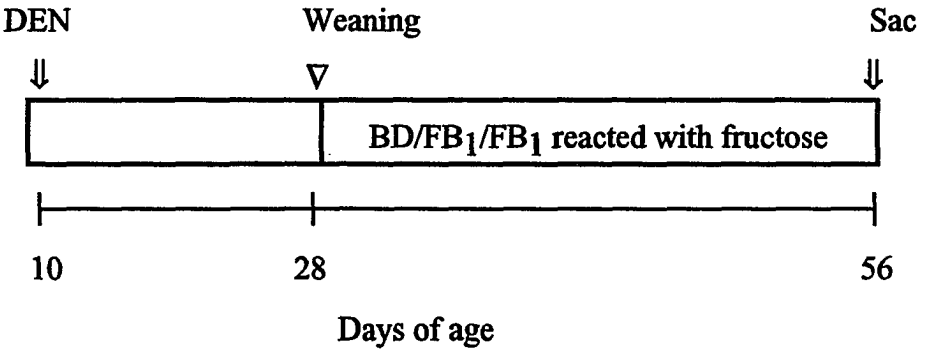


Figure 1. Schematic representation of the experiment with male F344/N rats fed fumonisin containing diet.

DEN: diethylnitrosamine, BD: basal diet, PB: phenobarbital, Sac: sacrifice

the same condition. At 56 days of age, all rats were killed by decapitation under CO_2 anesthesia.

Tissue Preparations

Liver samples were removed, freeze-clamped in liquid N_2 immediately and powdered, then stored at -70°C until analysis. Powdered liver (0.5 g) was homogenized with a Teflon-glass homogenizer in 5 ml of 20 mM HEPES (pH 7.4), containing 5 mM EDTA, 5 mM EGTA, 0.1 mM PMSF, 1 mM benzamidine and 4 μg/ml leupeptin. The homogenates were centrifuged at 4°C at 105,000xg for 1 hr to obtain supernatants.

Rabbit anti-rGST P1-1 antiserum and anti-CA III antiserum

Rat GST P1-1 was purified from rat liver as described by Satoh, K. *et al.* (1985) with the modification that the fractions were dialyzed with 10 mM dithiothreitol (DTT) overnight to protect sulfhydryl groups of the enzyme during purification. A mixture of 0.5 ml of rat GST P1-1 (100 μg) and 0.5 ml of Freund's complete adjuvant was subcutaneously administered to 4 spots on the back of one six month-old male New Zealand rabbit. A booster injection (0.5 ml of 1:1 mixture, 100 μg of rGST P1-1 and incomplete Freund's adjuvant) was administered 2 weeks after the initial injection. Blood was collected every other week from the central ear artery beginning one month after the first injection of the protein, allowed to clot, and centrifuged to prepared serum. Serum was stored frozen in aliquots at -70°C. The antiserum titer was checked by dot immunoassay (Otata & Cheng 1988) and 1:1000 dilution gave optimum detection of rGST P1-1 detection.

Rabbit anti-CA III serum was prepared previously in our laboratory (Lii et al. 1994) and CA III from rat liver was a gift from Dr. James A. Thomas in Biochemistry and Biophysics Department of Iowa State University (Chai et al. 1991).

Analysis of expression of rat GST P1-1 and CA III in male rat liver initiated with diethylnitrosamine and fed fumonisin containing diet

To examine and quantify the expression pattern of rGST P 1-1 and CA III, immuno-blot was performed as described by Towbin, H. *et al.*(1979). Proteins (10 µg/lane for rGST p1-1 and 0.2 µg/lane for CA III) were separated by SDS-PAGE (12% acrylamide), transferred to nitrocellulose membranes using a Bio-Rad mini trans-blot cell (Bio-Rad Lab., Hercules, CA) under constant 100 V and 1h running time. The nitrocellulose membrane was stained by the method of Obata and Cheng (1988) with some modification. The membrane was incubated in 1% BSA in 25 mM Tris buffer with 150 mM NaCl, pH 7.4 for 30 min at 37°C to block nonspecific binding of antiserum to membranes, and then, was incubated with a 1:1000 dilution of a rabbit anti-rGST P1-1 antiserum or a 1:500 dilution of a rabbit anti-CA III antiserum for 30 min at 37°C as primary antibody. An avidin/peroxidase and biotinylated anti-rabbit IgG kit (Vector Lab., Burlingame, CA) were used to detect immunoreactive protein bands. Each incubation with blocking buffer, primary antibody, secondary antibody, and avidin-peroxidase complex was 30 min at 37°C. Between

each step, membranes were washed 4 times for 5 min with buffer, pH 7.4, containing 25 mM Tris, 150 mM NaCl, and 0.1% Tween-20. A mixed solution of equal volume of 0.02% hydrogen peroxide in distilled H_2O and 0.1% 3,3'-diaminobenzidine in 0.1 M Tris buffer, pH 7.2 was used as peroxidase substrate for color development by treating membranes at room temperature for 15 min. The reaction was stopped by washing with water, and the membrane was air-dried.

In order to quantify expression of both enzymes, image analysis was done with a Zeiss-IPS image analysis system (Zeiss-Kontron, IBAS version 2.00). The amounts of rGST P1-1 and CA III were quantified by scanning of immuno-blot, and the proteins were quantified by comparison with known amounts of CA III and rGST P1-1. Statistical analysis was carried out using the Statistical Analysis System (Cary, NC). Differences between the groups were determined by using Student's *t*-test, where $p<0.05$ was taken as a statistically significant difference. And correlation between expression of rGST P1-1 and CA III was also determined.

RESULTS

Hepatic rGST P1-1 in male F344/N rat liver after DEN initiation and 1 month of promotion by fumonisin-containing diet differed significantly ($p < 0.05$) between the group treated with fumonisin alone and the control group. With the fumonisin treatment rGST P1-1 increased 980% over the amounts of this protein in the control rat livers (Table 1, Figure 2). Hepatic CA III levels also differed significantly between the fumonisin-treated group and the control group ($p < 0.05$). With fumonisin treatment, CA III decreased 43% over the amounts of this protein in the control rat livers (Table 1, Figure 2). There was a modest negative correlation between expression of rGST P1-1 and CA III ($r = -0.61$, $p < 0.0016$).

Levels of rGST P1-1 and CA III in rat liver treated with fumonisin reacted with fructose did not differ from the controls. Heating fumonisin with fructose may be a method for fumonisin detoxification, especially with regard to fumonisin carcinogenesis.

DISCUSSION

A disturbance in the redox state of cells due to reactive oxygen species (ROS) can generate oxidative stress. Proteins with reactive sulfhydryls are subject to oxidation

Table I. Hepatic π class rat glutathione S-transferase (rGSTP1-1) and carbonic anhydrase III (CA III) levels in liver tissue of male F344/N rats after 1 month of promotion by fumonisin B_1[a,b,c]

Group	n	CA III µg/mg protein	RGST P1-1 µg/mg protein
Control	6	166 ± 45[a]	0.6 ± 1.1[a]
Fumonisin	7	94 ± 24[b]	6.4 ± 1.8[b]
Fumonisin + Fructose	7	172 ± 41[a]	1.0 ± 0.8[a]

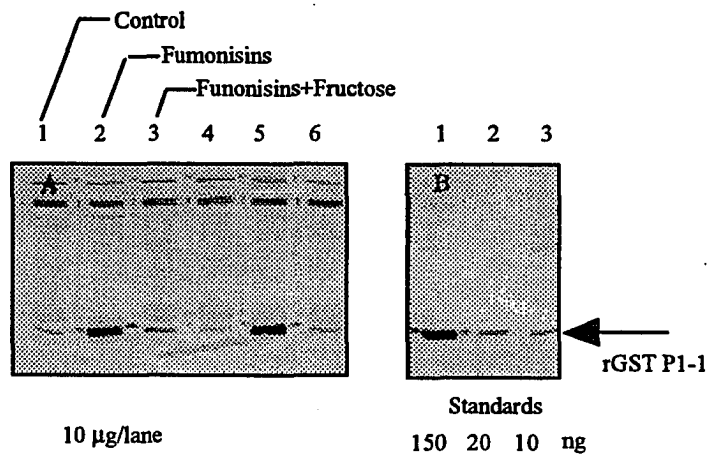
a: Values are means ± SD.

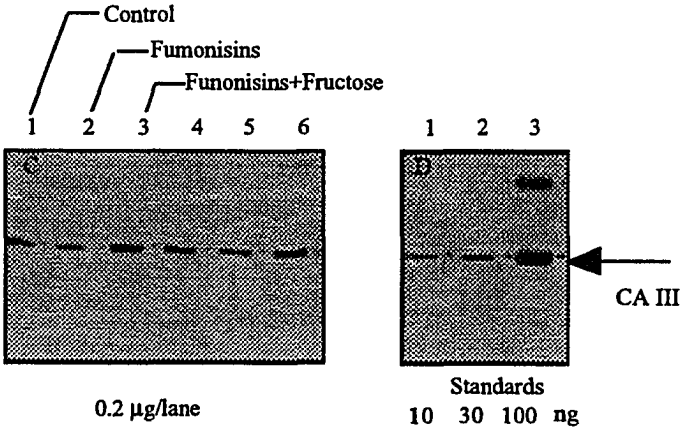
b: Groups not sharing a letter are significantly different ($p < 0.05$) by using Student's *t*-test.

c: The amounts of rGST P1-1 and CA III were quantified by scanning of immuno-blots, using anti-rGST P1-1 and anti-CA III antibodies, respectively and the proteins were quantified by comparison with known amounts of rGST P1-1 and CA III.

Figure. 2. Difference in expression of the π group of rat glutathione S-transferase P1-1(rGST P1-1) and carbonic anhydrase III (CA III) from the experiment using fumonisin containing diet

(A). Western blot of rat liver cytosolic PGST from the fumonisin experiment. *Lanes 1* and *4* Control; *2* and *5* Fumonisin; 3 and 6; Fumonisin reacted with fructose (10 μg protein each). (B) Western blot of purified rGST P1-1 for standards. *Lanes 1* 150 ng; *2* 20 ng; *3* 10 ng. (C) Western blot of rat liver cytosolic CA III from the fumonisin experiment. *Lanes 1* and *4* Control; *2* and *5* Fumonisin; *3* and *6* Fumonisin reacted with fructose (protein 0.2 μg each). (D) Western blot of purified CA III for standards. *Lanes 1* 10 ng; *2* 30 ng; *3* 100 ng.


Control
Fumonisins
Funonisins+Fructose
1 2 3 4 5 6
A
1 2 3
B
rGST P1-1
Standards
150 20 10 ng
10 µg/lane


Control
Fumonisins
Funonisins+Fructose
1 2 3 4 5 6
C
1 2 3
D
CA III
Standards
10 30 100 ng
0.2 µg/lane

and reduction during oxidative stress. Such proteins are abundant in cytosol, membranes, and mitochondria, and nuclei (Thomas & Sies 1992). Oxidative stress can lead to mixed disulfide bond formation through the covalent bonding of reactive protein sulfhydryls to low-molecular thiols such as GSH in a process called S-thiolation (Grimm et al. 1985). S-thiolated proteins can be reduced to their original state, with concomitant production of glutathione disulfide in a process called protein dethiolation (reduction) (Park & Thomas 1989). The evidence that reactive oxygen species produced during oxidative stress initiate S-thiolation of purified phosphorylase b, creatine kinase (Park & Thomas 1989, Miller et al. 1990), that the release of O_2^- from human neutrophil induced by phorbol-12-myristate-13-acetate (PMA) involves S-thiolation of various proteins including actin (Chai et al 1994b), and that these S-thiolated proteins return to their original forms by dethiolation or reduction process support a potential protective role of S-thiolation in oxidative stress.

In this experiment, the rGST P1-1 level increased 11 fold in the fumonisin treated-group compared with the control group. The content of rGST P1-1 in hyperplastic nodule-bearing livers and transplantable hepatomas increases 10 fold

over normal liver(Satoh et al. 1985), and that of human GST P1-1 in colon and esophageal cancer increases 6 fold compared with normal tissues (Tsuchida et al. 1989). Also our results showed that the level of CA III was 17% of total hepatic cytosolic protein in the control group, and the CA III level decreased 1.8 fold suggesting the possible involvement of this enzyme during rat hepatocarcinogeneis.

Although the reasons for an inverse relationship between expression of rGST P1-1 and CA III during male rat liver cancer development remain to be clarified, the fact that both enzymes contain reactive sulfhydryls (Dirr et al 1994, Thomas & Sies 1992) which can be modified through S-thiolation/dethiolation (Nishihara et al. 1991, Shen et al. 1993, Lii et al. 1994), and the occurrence of oxidative stress during tumor promotion (Sun 1990) leads us to propose that rGST and CA III increases during cancer development by fumonisins may play an important role in antioxidant function through protein S-thiolation/dethiolation. A possible detoxification process against fumonisin toxicity was introduced in which the amino group on fumonisin molecule was reacted with fructose, a reducing sugar, by browning or Maillard reaction.

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CHAPTER 2. SOYBEAN ISOFLAVONE EXTRACT SUPPRESSES EARLY BUT NOT LATE PROMOTION OF HEPATOCARCINOGENESIS BY PHENOBARBITAL IN FEMALE RAT LIVERS

A paper to be submitted to Nutrition and Cancer

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ABSTRACT The antioxidant and anticarcinogenic activities of soybean isoflavone extracts were investigated in female F344/N rats. Diethylnitrosamine (DEN, 15 mg/kg body weight) as a cancer initiator was injected intraperitoneally into 120 female F344/N rats at 10 days of age, and at weaning, phenobarbital (PB, 500 mg/kg diet) was fed to half of the rats. Soybean isoflavones were extracted in acetone/0.1N HCl and analyzed by HPLC, and two levels of isoflavones (920, 1840 μmol/kg diet) in crude soybean extract were fed during PB treatment for 3 and 11 months. Control rats were fed diets without PB and with or without isoflavones. The effect of soybean isoflavone extract on hepatic glutathione peroxidase (GPX) was measured, and development of γ-glutamyl-transferase (GGT)-positive and placental glutathione transferase (PGST)-positive altered hepatic foci (AHF) was analyzed by computerized stereology. Soybean isoflavone extract providing 920 or 1840 μmol/kg diet normalized total

hepatic glutathione peroxidase (tGPX) activity, which was suppressed about 17% by PB ($p<0.05$). Both doses of isoflavone extract suppressed PB promotion of hepatocarcinogenesis, decreasing the volume occupied by GGT-[+] AHF and PGST-[+] AHF ($p < 0.05$) after 3 months. After 11 months of PB promotion, 920 µmol isoflavone extract/kg diet decreased PGST-[+] AHF compared with the PB-fed group. But neither dose of isoflavone extract suppressed development of GGT-[+] AHF compared with the group fed PB alone. Furthermore, the control group fed 1840 µmol /kg diet of isoflavone extract showed greater development of GGT-[+] and PGST-[+] AHF than the group fed the basal diet alone. Therefore, soybean isoflavones may be anticarcinogenic, but their margin of safety is relatively narrow, with a cancer-promoting dose of 1840 µmol/kg in female F344/N rats initiated with DEN.

Introduction

The incidence of breast cancer in Asian women is much lower than in Western women (1,2). Japanese women and women of Japanese origin in Hawaii consuming a diet similar to the traditional Japanese diet had low breast cancer incidence and mortality (3). Dietary factors are suggested as a cause of the increasing breast cancer rate among successive

generations of Japanese in California as compared with the rate in Japan (2). Asian populations generally consume larger amounts of soybean foods than do populations in most Western countries (4,5).

Soybean consumption may prevent some types of cancer. Feeding a 30% soybean diet protects mouse liver from cancer development induced by nitrosamine precursors, dibutylamine and nitrite (6). Rats consuming a soy-based diet develop fewer mammary tumors following administration of the carcinogens N-methylnitrosourea and 7,12-dimethylbenz [a]-anthracene than rats on isonitrogenous and isocaloric diets without soy (7). In a case-control study of diet and breast cancer in Singapore Chinese, Lee *et al.* found that soy protein was associated with reduced risk in premenopausal women ($p < 0.02$), and suggested that the phytoestrogenic isoflavones in soy may be responsible (8). The anticarcinogenic effect of soy may come from biological activities of soybean isoflavones present in soybeans in amounts of approximately 1-3 mg/g (9). The isoflavones genistin, daidzin and their aglycones, genistein and daidzein, constitute 90-95% of the isoflavone content of soybeans (10).

Isoflavones have antioxidant activity according to results from methods such as determination of lipid peroxidation induction periods in which 0.1% genistein in lard at 100°C has distinct activity (11), measurement of relative rates of coupled oxidation of β-carotene and linoleic acid emulsion in which genistein and daidzein (5×10^{-4} M) have the activity (12), and judgment of ability to inhibit the activity of lipoxygenase in which 5×10^{-5} M genistein shows 30% inhibition of soy bean lipoxygenase compared with control (13). This function of isoflavones as antioxidants may be critical to the anticarcinogenic effects of these compounds. Reactive oxygen species (ROS) (such as hydroxyl radicals (•OH), superoxide anion radicals ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2)), and their subsequent modification of macromolecules (such as protein, RNA, and DNA), may be involved in development of multi-stage carcinogenesis, particularly in tumor promotion (14-17).

Phenobarbital (PB) is a potent tumor-promoting agent (18) and can cause oxidative damage to liver (19). Neoplastic rat liver nodules in which rats were initiated with a single injection of diethylnitrosamine (i.p. 0.15 μmol/kg body weight) and promoted by 0.05% PB in diet show increased

levels of ROS, which was detected in microsomal fractions from the different tissues by measuring NADPH-dependent lucigenin chemiluminescence, compared with the tissue from normal liver and surrounding normal tissue of the nodule-containing liver. The generation of ROS is attributed to the induction of certain cytochrome P-450 isoenzymes by PB treatment, which was detected by anti cytochrome P-450 PB4/PB5, antibody (20). Antioxidants may protect cells from oxidative damage (17). Antioxidant effects which may inhibit tumor promoter-induced oxidant formation and inflammatory responses could lead to anticarcinogenic activities of isoflavones. In addition to antioxidant activity, isoflavones also are estrogenic or antiestrogenic depending on their dose (21-26). The fact that isoflavones and estrogen can compete for estrogen-receptor complex may explain estrogenic and antiestrogenic effects of isoflavones (25,26). The antiestrogenic and antioxidant activities of isoflavones and their inhibitory effects on several enzymes such as tyrosine-specific protein kinases (TPK) (27), casein kinase II (28), DNA topoisomerase (29,30), and S6 kinases (27), which are involved in cell proliferation, indicate that isoflavones at some doses may contribute to the prevention of many types of cancer.

Carcinogenesis in both mouse skin (31) and rat liver models (32,33) consists of three stages: initiation, promotion, and progression. Hepatocarcinogenesis in the rat provides a readily quantifiable multistage model of cancer development. γ-Glutamyltransferase (GGT) and placental glutathione transferase (PGST) are useful biomarkers of hepatocarcinogenesis (34-36). The combination of these two markers can score most of the altered hepatic foci (AHF) that develop during phenobarbital promotion after initiation by diethylnitrosamine (DEN) with PGST being the better (36) because PGST may specifically identify more rapidly growing preneoplastic lesions (Hendrich et al. 1987) and more persistent of the two markers (37).

Previous studies of cancer protection by soybeans using animals have not fed specific soy components that may account for anticancer effects, and have been done over relatively short time periods. Cancer-related studies using pure isoflavones have been performed with cell lines *in vitro* and not *in vivo*. In this study, we have examined hepatocarcinogenesis which was induced in female F344/N rats by diethylnitrosamine (DEN) initiation and PB promotion. This study includes measurement of glutathione peroxidase (GPX) activity, which may be an index of the body's capacity

to detoxify peroxides, and quantification of altered hepatic foci (AHF) with GGT and PGST as markers. The rats were fed basal diet or diet containing soybean isoflavone extract for 3 and 11 months.

Materials and Methods

Animals and diets

The experimental protocol was reviewed and approved by the Iowa State University Animal Use Committee. Female F344/N rats were obtained from Harlan/Sprague-Dawley (Indianapolis, IN). The animals were housed in a room maintained at 22°C with lighting 0600 to 1800h. They were fed and given tap water *ad libitum*. Body weight and food consumption were recorded weekly. Soybean isoflavone extract was prepared from toasted, defatted soyflakes (Ames Feed & Supply, Inc., Ames IA) by extraction. Briefly, soyflakes were extracted by stirring in acetone : 0.1N HCl (5:1 v/v) in which flakes are added to the solvent (g/ml). After filtration through Whatman filter paper #42, the extract was concentrated on a steam bath, then concentrated by using a rotary evaporator at 30°C and freeze dried. The isoflavone content of the extract was measured as described by Wang and Murphy (38).

Total daidzein and genistein constituted 3.77 and 1.81% of the extract. The extract was mixed into basal diets to

provide 920 and 1840 μmol total isoflavones/kg diet. The basal diet contained 40 % of kcals from fat and was otherwise based upon the standard AIN-76 rat diet (Table 1).

Experimental Design

At ten days of age, 12 litters of 10 female F344/N rats were dosed by intraperitoneal injection with diethylnitrosamine (DEN, 15 mg/kg) as a cancer initiation agent. At 4 weeks of age, weaned pups were fed basal diets, and were randomly divided into six groups fed with or without 0.05% PB and with or without 920 or 1840 μmol total isoflavones/kg diet. Rats were killed at 3 and 11 months after weaning for examining the preneoplastic and neoplastic development of hepatocarcinogenesis respectively.

Analytical procedures

Chemicals were obtained from Sigma Chemical (St. Louis, MO) unless specified. Rats were killed by decapitation under CO_2 anesthesia. The livers were homogenized in a 50 mM potassium phosphate buffer (pH 7.0). The homogenates were centrifuged at 4°C at 105,000g for 1 hr to obtain the supernatants. Protein was determined using the Bio-Rad protein reagent (Bio-Rad Laboratories, Richmond, CA). Glutathione *S*-transferase activity of the liver cytosolic fraction was determined in the presence of 1.0 mM GSH with

Table 1. Composition of the diets (g/kg)[a,b]

	Diet		
Ingredients	Basal	Total Isoflavone Content	
		920 µmol/kg diet	1840 µmol/kg diet
g/kg			
Beef Tallow[a]	139.6	139.6	139.6
Corn Oil[b]	66.5	66.5	66.5
Casein[a]	224.1	224.1	224.1
Corn Starch[a]	228.5	228.5	228.5
Dextrose[a]	224.1	219.8	215.6
Cellulose[a]	56.0	56.0	56.0
AIN Vit Mix 76[a]	11.2	11.2	11.2
AIN Mineral Mix 76[a]	39.2	39.2	39.2
$CaCO_3$[a]	5.0	5.0	5.0
Choline Bitartrate[a]	2.2	2.2	2.2
D,L-Methionine[a]	3.04	3.04	3.04
Ascorbate[a]	0.112	0.112	0.112
Isoflavone Extract	-	4.3	8.5

a: Teklad, Madison, WI.

b: Iowa State University Food Stores, Ames, IA

1.0 mM 1-chloro-2,4-dinitrobenzene as the cosubstrate. The enzyme activity was measured spectrophotometrically (39). Hepatic total glutathione peroxidase activity (tGPX), measured using cumene hydroperoxide as a substrate and hepatic selenium-dependent glutathione peroxidase activity (sGPX), measured using hydrogen peroxide as a substrate, were determined by the method of Lawrence and Burk (40). The reaction system contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 enzyme unit of glutathione reductase, 1 mM reduced glutathione, and 1.5 mM cumene hydroperoxide or 0.25 mM hydrogen peroxide. The three largest lobes of the liver were sliced into 0.5 cm slices, and three slices, one from each of the lobes, were frozen together as a block on dry ice for preparation of serial sections at 3 and 11 months after weaning. The frozen sections were stained for (GGT) activity and the presence of PGST protein. The GGT sections were mounted on slides and air dried and then stained according to the method of Kalengayi *et al.* (35). For PGST staining, the second frozen sections were air dried and fixed in acetone:ethanol (1:1) for 3 min, and the slides were incubated overnight in anti-PGST antiserum (1:500) at 4°C. The PGST was detected as described by Hendrich *et al.* (41). The GGT- and PGST-

positive altered hepatic foci (AHF) were quantified by computerized stereology according to Campbell *et al* (42), employing an HP 9845B computer, an HP 9845A disk drive (10-inch diskettes), an HP 9842C plotter (Hewlett-Packard, Cupertino, CA), an LA120 printer (Digital Equipment, Maynard, MA), and a Microgrid II digitizer (Summagraphics, Fairfield, CT). The PGST was purified from rat liver as described by Satoh, K. *et al.* (34) with some modification, that is, after each column the fractions were dialyzed with 10 mM dithiothreitol (DTT) overnight to protect sulfhydryl groups of the enzyme. Rabbit anti-PGST antiserum was prepared as follows; 100 μg of purified PGST was injected subcutaneously (SC) in a 1:1 mix with Freund's complete adjuvant (0.5 ml) into one 12 month-old male rabbit, and a 100 μg booster injection in a 1:1 mix with Freud's incomplete adjuvant (0.5 ml) was injected SC 2 weeks after the first injection. The antiserum was prepared by biweekly bleeding from the ear vein beginning 1 month after the initial injection of antigen.

Statistical analysis

Statistical analysis was carried out using the Statistical Analysis System (Cary, NC). Data were expressed as means ± SD. Differences between the groups were

determined using Student's *t*-test, where $p < 0.05$ was taken as a statistically significant difference.

Results

After three months of treatment

Body weight, Relative liver weight, and Food intake

No differences ($p < 0.05$) among groups were found in body weights and average daily food intake at either time point (data not shown). Phenobarbital significantly ($p < 0.05$) increased relative liver weight by 33% and plasma cholesterol by 38% compared with control rats. (Table 2).

Enzyme activities

Hepatic cytosolic glutathione *S*-transferase activity was increased by 100% by PB treatment (Table 2) ($p < 0.05$), and hepatic total cytosolic GSH peroxidase (tGPX) and selenium-dependent GSH peroxidase (sGPX) activities were decreased by PB treatment compared with the control group at 3 months (Figure 1). Total GPX activity, which was suppressed 17% by PB, was normalized in PB-fed groups by soybean isoflavone extract feeding whereas sGPX activity was not normalized in PB-fed groups by isoflavone extract feeding (Figure 1).

Histochemistry

Phenobarbital significantly increased the percentage of hepatic volume occupied by GGT-[+] AHF and PGST-[+] AHF,

Table 2. Effects of soy isoflavone extract and phenobarbital on systemic parameters in female F344/N rats after 3 months of treatment[a-c]

Treatment[c]	n	Relative Liver/Body Weight, %	Total Plasma Cholesterol mg/dl	Glutathione *S*-transferase activity µmole/mg/min
Control	8	3.0 ± 0.2[a]	108.9 ± 15[a]	446 ± 76[a]
PB	8	4.0 ± 0.2[b]	144.5 ± 9.4[b]	905 ± 105[b]
Iso_{920}	8	3.0 ± 0.2[a]	104.8 ± 12[a]	421 ± 76[a]
Iso_{920} + PB	8	4.0 ± 0.2[b]	141.8 ± 14[b]	921 ± 85[b]
Iso_{1840}	8	3.0 ± 0.2[a]	101.6 ± 8.8[a]	466 ± 37[a]
Iso_{1840} + PB	8	3.9 ± 0.2[b]	143.5 ± 18[b]	888 ± 100[b]

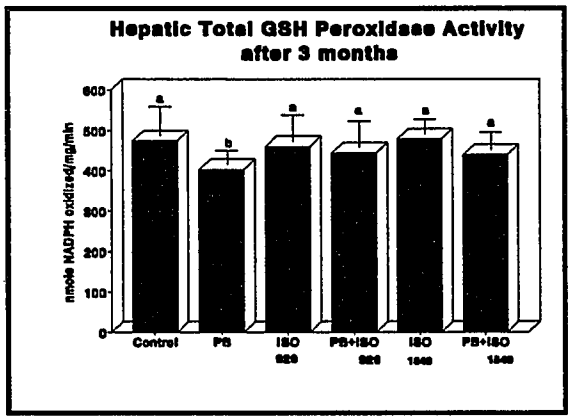
a: Values are means ± SD.

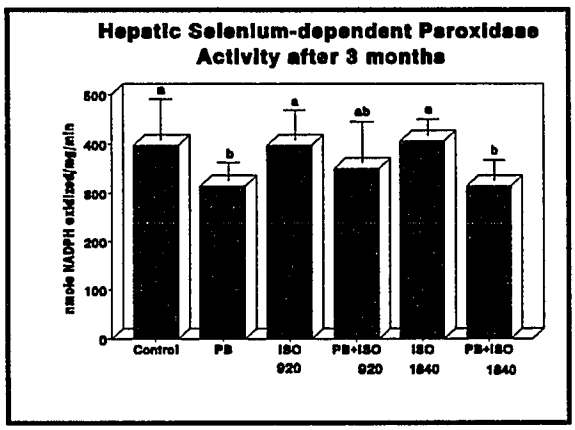
b: Groups not sharing a letter are significantly different ($p < 0.05$).

c: Iso_{920}/ Iso_{1840}, soybean isoflavone extract containing diet (920 µmol/kg diet or 1840 µmol/kg diet); PB: phenobarbital (500 mg/kg diet).

Figure 1. Female F344/N rat hepatic total GSH peroxidase (tGPX) and selenium-dependent GSH peroxidase (sGPX)activities of soy isoflavone extract and phenobarbital after 3 months of treatment

Groups not sharing a letter are significantly different ($p < 0.05$). Iso_{920}/ Iso_{1840}, soybean isoflavone extract containing diet (920 µmol/kg diet or 1840 µmol/kg diet); PB: phenobarbital (500 mg/kg diet).


Hepatic Total GSH Peroxidase Activity
after 3 months
nmole NADPH oxidized/mg/min
600
500
400
300
200
100
0
a
b
a
a
a
a
Control
PB
ISO
920
PB+ISO
920
ISO
1840
PB+ISO
1840


Hepatic Selenium-dependent Peroxidase
Activity after 3 months
nmole NADPH oxidized/mg/min
500
400
300
200
100
0
a
b
a
ab
a
b
Control
PB
ISO
920
PB+ISO
920
ISO
1840
PB+ISO
1840

about 3 fold and 13 fold, respectively, compared with the control group (Table 3). Both concentrations of soybean isoflavone extract (920 or 1840 μmol/kg diet) significantly ($p<0.05$) suppressed PB's ability to increase development of GGT-[+] AHF and PGST-[+] AHF (Table 3). There were no significant differences in number of GGT-[+] AHF per cm^2 among the groups (Table 4). On the other hand, the number of PGST-[+] AHF per cm^2 was increased about 5 fold by PB treatment compared with the control group, whereas both concentration of soybean isoflavone extract significantly($p<0.05$) suppressed the number of PGST-[+] AHF per cm^2 by PB treatment.

After eleven months of treatment

Body weight, Relative liver weight, and Food intakes

No significant difference ($p<0.05$) among groups were found in average daily food intake (data not shown). PB significantly ($p<0.05$) decreased body weight by 11%, and increased relative liver/body weight ratio by 50% (Table 5).

Enzyme activities

Hepatic cytosolic tGPX and sGPX activities were significantly decreased 29% and 54%, respectively by PB treatment (Figure 2). Isoflavone extract did not significantly alter PB's effect on peroxidase activities

Table 3. Effects of soy isoflavone extract and phenobarbital on development of gamma-glutamyl-transferase- and placental glutathione S-transferase-positive hepatic foci in female F344/N rat liver after 3 and 11 months of treatment[a-c]

Treatment[c]	% of liver volume occupied by GGT-[+] foci		% of liver volume occupied by PGST-[+] foci	
	3 months	11 months	3 months	11 months
Control	0.07 ± 0.06^{b}	0.13 ± 0.09^{c}	0.03 ± 0.02^{b}	0.03 ± 0.01^{c}
PB	0.22 ± 0.12^{a}	0.52 ± 0.22^{a}	0.38 ± 0.17^{a}	0.33 ± 0.13^{a}
Iso_{920}	0.04 ± 0.02^{b}	0.24 ± 0.12^{bc}	0.03 ± 0.01^{b}	0.11 ± 0.12^{bc}
Iso_{920} + PB	0.04 ± 0.02^{b}	0.47 ± 0.19^{a}	0.10 ± 0.07^{b}	0.10 ± 0.04^{b}
Iso_{1840}	0.02 ± 0.02^{b}	0.29 ± 0.15^{b}	0.05 ± 0.05^{b}	0.18 ± 0.10^{ab}
Iso_{1840} + PB	0.12 ± 0.08^{b}	0.49 ± 0.21^{a}	0.05 ± 0.05^{b}	0.21 ± 0.09^{ab}

a: Values are means ± SD.

b: Groups not sharing a letter are significantly different ($p < 0.05$).

c: Iso_{920}/ Iso_{1840}, soybean isoflavone extract containing diet (920 µmol/kg diet or 1840 µmol/kg diet); PB: phenobarbital (500 mg/kg diet).

Table 4. Effects of soy isoflavone extract and phenobarbital on gamma-glutamyltransferase - and placental glutathione S-transferase - positive altered hepatic foci (AHF) per sq cm in female F344/N rat liver after 3 and 11 months of treatment[a-c]

Treatment[c]	GGT lesion transections/sq cm		PGST lesion transection/ sq cm	
	3 months	11 months	3 months	11 months
Control	0.77 ± 0.23^{a}	0.51 ± 0.27^{c}	1.38 ± 0.48^{c}	0.85 ± 0.66^{b}
PB	2.13 ± 1.95^{a}	1.32 ± 0.42^{ab}	6.61 ± 2.81^{a}	2.59 ± 0.80^{a}
Iso_{920}	1.66 ± 0.22^{a}	0.69 ± 0.28^{c}	1.37 ± 1.02^{bc}	2.11 ± 0.66^{a}
Iso_{920} + PB	0.69 ± 0.27^{a}	1.13 ± 0.62^{bc}	2.60 ± 0.95^{b}	1.95 ± 0.86^{ab}
Iso_{1840}	0.88 ± 0.54^{a}	0.68 ± 0.36^{c}	1.41 ± 0.59^{bc}	1.28 ± 0.81^{ab}
Iso_{1840} + PB	1.73 ± 0.58^{a}	1.19 ± 0.66^{b}	1.72 ± 0.30^{bc}	2.81 ± 1.39^{a}

a: Values are means ± SD.

b: Groups not sharing a letter are significantly different ($p < 0.05$).

c: Iso_{920}/ Iso_{1840}, soybean isoflavone extract containing diet (920 µmol/kg diet or 1840 µmol/kg diet); PB: phenobarbital (500 mg/kg diet).

Table 5. Effects of soy isoflavone extract and phenobarbital on systemic parameters in female F344/N rats after 11 months of treatment[a-c]

		Body Weight, g	Relative Liver/Body Weight, %
Treatment[c]	n	Final	
Control	12	284.4 ± 21.4[a]	3.0 ± 0.2[a]
PB	12	255.8 ± 14.9[b]	4.5 ± 0.4[b]
Iso_{920}	11	291.6 ± 24.3[a]	3.1 ± 0.3[a]
Iso_{920} + PB	12	264.5 ± 24.0[b]	4.2 ± 0.3[b]
Iso_{1840}	11	290.5 ± 17.0[a]	3.1 ± 0.1[a]
Iso_{1840} + PB	12	249.7 ± 26.1[b]	4.4 ± 0.4[b]

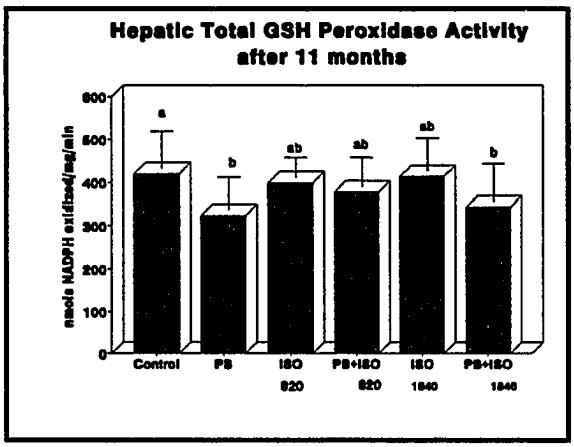
a: Values are means ± SD.

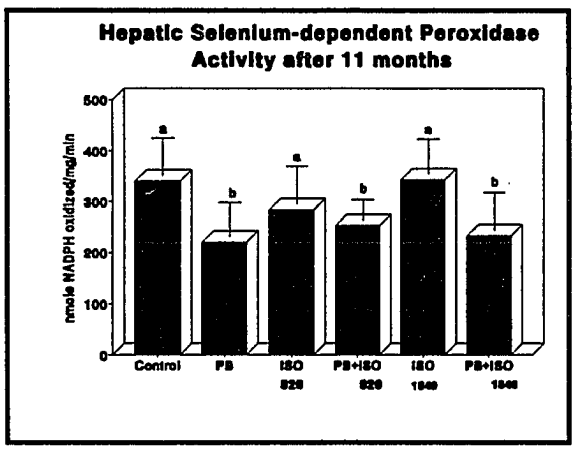
b: Groups not sharing a letter are significantly different ($p < 0.05$).

c: Iso_{920}/ Iso_{1840}, soybean isoflavone extract containing diet (920 μmol/kg diet or 1840 μmol/kg diet); PB: phenobarbital (500 mg/kg diet).

Figure 2. Female F344/N rat hepatic total GSH peroxidase (tGPX) and selenium-dependent (sGPX)activities of soy isoflavone extract and phenobarbital after 11 months of treatment.

Groups not sharing a letter are significantly different ($p < 0.05$). Iso_{920}/ Iso_{1840}, soybean isoflavone extract containing diet (920 µmol/kg diet or 1840 µmol/kg diet); PB: phenobarbital (500 mg/kg diet).


Hepatic Total GSH Peroxidase Activity after 11 months
nmole NADPH oxidized/mg/min
600
500
400
300
200
100
0
a
b
ab
ab
ab
b
Control
PB
ISO 820
PB+ISO 820
ISO 1640
PB+ISO 1640


Hepatic Selenium-dependent Peroxidase Activity after 11 months
nmole NADPH oxidized/mg/min
500
400
300
200
100
0
a
b
a
b
a
b
Control
PB
ISO 820
PB+ISO 820
ISO 1640
PB+ISO 1640

although the group fed 920 µmol isoflavone extract/kg diet with PB increased tGPX activity by 17 % (Figure 2).

Histochemistry

Phenobarbital significantly increased the percentage of hepatic volume occupied by GGT-[+] AHF and PGST-[+] AHF 4 fold and 11 fold, respectively compared with the control group (Table 3). Isoflavone extract did not suppress PB's effect to promote GGT-[+] AHF. Although isoflavone extract did not suppress development of PB-promoted GGT-[+] foci, 920 µmol isoflavone extract/kg diet fed with PB compared with rats fed PB alone decreased PGST-[+] AHF. The group fed 1840 µmol isoflavone extract/kg diet without PB showed greater development of GGT-[+] and PGST-[+] AHF than did the control group. The numbers of GGT- and PGST-[+] AHF per cm^2 promoted by PB treatment was significantly increased compared with the control group (Table 4). Isoflavone extract did not decrease numbers of GGT-[+] and PGST-[+] AHF per cm^2 promoted by PB. The group fed 920 µmol isoflavone extract/kg diet without PB had a greater number of PGST-[+] AHF section/cm^2 than the group fed the basal diet alone.

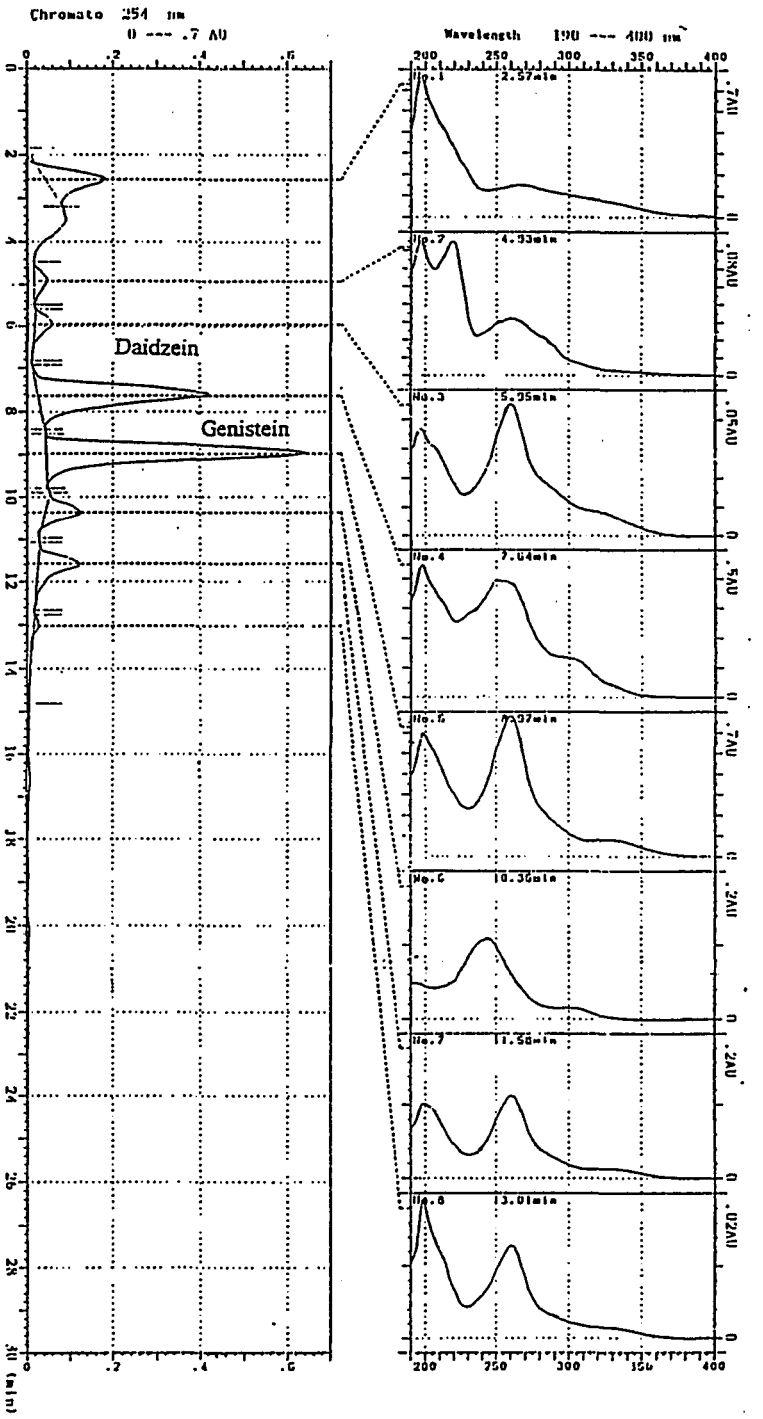
Discussion

Because the soyflakes used for the isoflavone extract were toasted, and extracted with acetone/0.1N HCl, protease inhibitors such as the Kunitz trypsin inhibitor were not likely to be extracted into acetone. The soyflakes had been commercially defatted, which eliminated most lipophilic components from the extract. Soybean seeds contain 10% soluble carbohydrates consisting of 5% sucrose, 1% raffinose, and 4% stachyose (43), and the quantity of the soybean isoflavones is about 1-3 g/kg in soybeans (9). The HPLC chromatogram of isoflavone extract used in this study (Figure 3) indicated that 32.6% and 24.7%of the UV absorbing portion were contributed by daidzein and genistein, respectively. The concentrations of daidzein and genistein in soybean extract used in this study were 3.77% and 1.81% of the extract, respectively. Although the glucosides have much less antioxidant activity judged by ability to inhibit the activity of lipoxygenase (13), the glucosides are cleaved in the gut and only aglycones absorbed by the body (44).

The present study confirmed that PB in the dose given exerted hepatotoxic effects leading to an increase in relative liver weight and total plasma cholesterol concentration at 3 months of treatment (Table 2)

Figure 3. HPLC retention profiles and spectra of soybean extract.

Soybean extract was prepared, and analyzed by HPLC as described in **Materials and Methods.** The retention profiles and absorbance spectra were obtained with a Waters 991 Photodiode Array Detector (Waters, Marlborough, MA).


Chromato 254 nm
0 --- .7 AU
Wavelength 190 --- 400 nm
Daidzein
Genistein
No.1 2.57min
No.2 4.93min
No.3 5.95min
No.4 7.04min
No.5 7.97min
No.6 10.36min
No.7 11.58min
No.8 13.01min
.7AU
.5AU
.05AU
.2AU
.02AU
(min)

and to a decrease in body weight at 11 months of treatment (Table 4). This study confirmed the ability of PB to promote hepatocarcinogenesis (45, Tables 3 and 4).

An indirect antioxidant activity of isoflavones may play an important role for anticarcinogenic effects of these compounds. Glutathione peroxidase (GPX) is part of the body's free radical scavenger system, which protects from oxygen-induced increases in levels of peroxides (36,46-50). After 3 months of treatment, isoflavone feeding normalized hepatic cytosolic total GPX (tGPX) activity which was suppressed 17% by PB, while PB-decreased hepatic cytosolic Se-dependent GPX (sGPX) activity was not affected by isoflavone extract feeding, and isoflavone extract alone did not affect GPX (Figure 1).

Because several types of glutathione (GSH) transferases have Se-independent GPX activity (51,52), the fact that isoflavone extract increased total GPX activity only during PB cotreatment may be due to an effect on specific PB-induced GST isoforms (Table 2). After 3 months of treatment, both doses of isoflavone extract suppressed development of GGT-[+] AHF and PGST-[+] AHF by PB, whereas isoflavone extract had less anticarcinogenic effect after 11 months of treatment, with only the dose of 920 μmol isoflavone extract/kg diet

decreasing PGST-[+] AHF development by PB (Table 3). This suggests that the anticarcinogenic action of isoflavones is directly related to isoflavone effects on tGPX. Also, significantly increased activity of hepatic total tGPX by isoflavone extract (at 920 μmol isoflavones/kg diet) fed to rats for one week (data not shown) suggests a short-term marker of biological effects of isoflavones which may predict anticancer effect of the compounds at later stage (53).

The facts that neither dose of isoflavone extract decreased GGT-[+] AHF at 11 months, and the high dose of isoflavones stimulated AHF that had not been PB-promoted suggest that several things might have happened. One is that after 11 months of treatment the PB-promoted precancerous cells have progressed to a later stage in their development where isoflavones are no longer effective. Seemingly, this stage is marked to a greater extent by GGT than PGST. Isoflavone-insensitivity of GGT-[+] AHF might also be due to decreased availability of isoflavones to these cells. It may also be that a new isoflavone-stimulated preneoplastic cell type is appearing later in life. Our study indicates that there might be two different cell types: PB-promoted or PB-independent, on which isoflavone extracts have different effects at 11 months.

A possible explanation for the later carcinogenic effects of isoflavones may be related to the antiestrogenic effects of these compounds. Genistein, at a dose of 200 mg/kg body weight for 4 days increases uterine weight in mouse compared with controls (21). The rats used in our experiment consumed about 36 mg/kg body weight per day, a dose much less than that causing an estrogenic effect in mice. Therefore, in amounts given in our study, isoflavones are probably not estrogenic and may act as antiestrogens. In addition, the uterotrophic effects of multiple injection of the daidzein metabolite, equol or of equol plus estradiol are lower than that of estradiol alone. Thus, equol and the isoflavones may act as antiestrogens (25).

Antiestrogenic effects of isoflavones might suppress breast cancer development, as does the antiestrogen, tamoxifen (TAM). But perhaps with long term administration, isoflavones may be hepatocarcinogenic in some doses, as is TAM. After one year of dosing, 2.8 mg TAM/kg/day is not hepatocarcinogenic, whereas 11.3 or 45 mg TAM/kg/day causes hepatocellular adenomas and carcinomas in female Sprague-Dawley rats. 45 mg TAM/kg/day causes the same types of hepatocellular alterations after 6 months exposure (54). Further support for the concept that an antiestrogenic

regimen may be hepatocarcinogenic comes from a study of hepatocarcinogenesis in ovariectomized rats. DEN-initiated ovariectomized rats, after withdrawal and reintroduction of phenobarbital, had significantly greater development of AHF than their sham-operated counterparts (55).

The fact that soybean extract actually increased development of AHF after 11 months in rats not promoted with PB, coupled with the possibilities discussed above, indicates that further study is required. A way to determine the balance of positive and negative effects of individual isoflavones or their combinations is needed. Also, well controlled animal studies are needed in which a continuous diet of a mixture of the major isoflavones as well as each isoflavone separately are fed to identify a combination and dosage of isoflavones which are safe and cancer-preventive. The margin of safety of these compounds may be quite narrow.

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CHAPTER 3. CHARACTERIZATION OF *S*-THIOLATION OF THE π CLASS OF RAT GLUTATHIONE *S*-TRANSFERASE

A paper to be submitted to Archives of Biochemistry and Biophysics

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ABSTRACT The proposed function of protein S-thiolation and dethiolation as an antioxidant system may play an important role during oxidative stress. In this paper, the characterization by isoelectric focusing (IEF) gel/SDS-PA gel electrophoresis and immuno-blot methods of S-thiolation/dethiolation and protein-protein disulfide bond formation of the π class of rat glutathione *S*-transferase (rGST P1-1) was performed with 1) purified rGST P1-1; 2) male Sprague-Dawley rat liver extract containing large amounts of rGST P1-1, as induced by a modified Solt-Farber protocol; 3) cultured rat hepatocyte extract containing rGST P1-1 induced by 2.5 mM sodium butyrate treatment. The reaction of rGST P1-1 with 20 mM GSSG for 1 or 3 h gave only partially S-thiolated bands, whereas when the enzyme was reacted with GSSG for 24h, rGST P1-1 showed thiolated bands and no fully reduced band. In addition, 8 and 24 h incubations with 20 mM GSSG showed a pattern of S-thiolation similar to that obtained after 5 min incubation with 4 or 6 mM diamide,

providing further support to the concept that rGST P1-1 contains thiolatable sulfhydryls.

The combined use of IEF and SDS-PAGE could directly detect the time dependent S-thiolation of rGST P1-1 to form a dimer between its subunits, as stimulated by 6 mM diamide in reaction with hepatic cytosolic extract from rats treated with a modified Solt-Farber protocol to rapidly induce rGST P1-1 -positive altered hepatic foci. Five forms of rGST P1-1 were identified: *band a*, fully reduced; *band b*, mono-glutathiolated; *band c*, mono-glutathiolated disulfide; *band d*, di-glutathiolated; *band e*, di-glutathiolated disulfide form of rGST P1-1. The proposed thiol modifications alter rGST P1-1 catalytic activity because molecular modeling of the three-dimensional structure of GST P1-1 indicated that Cys 47, one of the most reactive sulfhydryls is near the active site of the enzyme. Altered rGST P1-1 catalytic activity might occur during oxidative stress associated with cancer development or antineoplastic drug metabolism.

The proposed role of protein S-thiolation/dethiolation and protein-protein disulfide bond formation as an antioxidant system may extend to this π class enzyme, which

could protect preneoplastic and neoplastic cells from oxidative stress during their growth.

INTRODUCTION

The superfamily of glutathione *S*-transferases (GSTs) (EC 2.5.1.18) are phase II enzymes, which conjugate glutathione with a variety of reactive electrophiles by catalyzing the nucleophilic attack of the sulfur atom of glutathione on electrophilic groups in a second substrate (Mannervik & Danielson 1988). According to substrate specificities, sensitivities to certain inhibitors, cross-reactivities with specific antibodies, isoelectric point, and N-terminal amino acid sequences, glutathione *S*-transferases may be grouped into at least four species-independent gene classes, termed alpha, mu, pi, theta of cytosolic enzymes, and a class of microsomal enzymes (Tsuchida & Sato 1992). A recent nomenclature system for human glutathione *S*-transferases, which can be also applied to other mammalian species, has been proposed (Mannervik et al. 1992). This new nomenclature system for glutathione *S*-transferases (GST), uses for example, acronyms hGST A1-1, rGST M1-1, pGST P1-1 etc., in which the prefix indicate the species (h, human; r, rat; m, mouse; p, porcine; b, bovine; rb, rabbit; c, chicken; gp, guinea pig); A, M and P indicating gene class alpha, mu

and pi; 1-1 indicating a dimer of two type-1 subunits (Dirr et al. 1994). The cytosolic glutathione *S*-transferases are dimeric proteins which can be noncovalently assembled as combinations of subunits, about 22-26.5 kDa each, from the same gene class (Mannervik & Danielson 1988). The cytosolic homodimeric π class of glutathione *S*-transferases (GST P1-1) whose subunit is 24-24.5 kDa, are known to be early and persistent markers of carcinogenesis. The expression of GST P1-1 is associated with the development of anticancer drug resistance (Coles & Ketterer 1990, Morrow & Cowan 1990). This enzyme detoxifies organic hydroperoxides and lipid hydroperoxides (Mannervik & Danielson 1988). The sequence of cysteine residues on subunits of GST P1-1 are highly conserved according to the reported amino acid sequences indicating the importance of their catalytic activity. The π class of glutathione *S*-transferases from human (hGST P1-1), rat (rGST P1-1), bovine (bGST P1-1) and mouse (mGST P1-1) in which the subunits of GST P1-1 have 209 amino acid residues, and has four cysteine residues per monomer at 14, 47, 101 and 169 on their subunits except mouse subunit of mouse GST P1-1 containing three Cys 14, 47 169 residues (Kano et al 1987, Okuda et al 1987, Hernando et al 1992, and Hatayama et al

1990). In addition, pig GST P1-1 has a subunit consisting of 207 amino acid residues lacking Gln 40 and Gly 41 of hGST P1-1 and rGST P1-1 (Dirr et al. 1994, Tamai et al. 1990). Because of the extensive sequence homology within the π class, Cys 45, 99, and 167 in pig sequence most likely correspond with Cys 47, 101, and 169 in human and rat sequences (Dirr et al. 1994, Rokutan et al 1989).

The identification of the reactive sulfhydryls on the subunits of GST P1-1 using selective modifiers of thiol groups of the protein, followed by endopeptidase cleavage, HPLC separation and amino acid sequence analysis shows different results because of the chemical reactivities or stabilities of the adducts between sulfhydryl modifiers and cysteine residues. Cys 47 of hGST P1-1, rGST P1-1 and mGST P1-1 subunits is selectively labeled when N-maleimide derivatives such as N-(anilino-1-naphtyl) maleimide (ANM) and N-ethylmaleimide (NEM) are used for sulfhydryl modification (Bello et al. 1990, Tamai et al. 1990), whereas Cys 47 and Cys 101 of rGST P1-1 subunit are two highly reactive thiols when 7-fluoro-4-sulfamoyl-2,1,3-benzodiazole (ABD-F) and methylmethanethio-sulfonate are used as (Nishihira et al. 1992). Also when the reduced form of hGST P1-1 is reacted with thiol specific agents such as 0.1 mM 5,5′-dithiobis-(2-

nitrobenzoic acid) (DTNB) or 0.15 mM 4,4'-dithiodipyridine (DTDP), the enzyme has two fast reacting sulfhydryls/subunit both titrable with DTNB and DTDP (Ricci et al. 1991). Thus, the π class of glutathione *S*-transferases seem to have two most reactive sulfhydryls of Cys at the 47th and 101st (the 45th and 99th in pGST P1-1) from the N-terminus of their subunit except mouse GST P1-1 which possess Gly instead of Cys at the 101st residue. Also, the three dimensional structure of pGST P1-1 suggests that Cys 45 and Cys 99 corresponding to Cys 47 and Cys 101 of hGST P1-1 and rGST P1-1 are not located directly in the GSH binding site but are in proximity to the active site of the enzyme (Dirr et al. 1994).

The role, if any, of the sulfhydryls present in each subunit of the π class is not fully understood. During oxidative stress the protein sulfhydryls are involved in "protein S-thiolation" (formation of mixed disulfide between protein sulfhydryls and low molecular thiols, such as GSSG)/ "dethiolation"(reduction of the mixed disulfide to sulfhydryls) (Chai et al 1994 a and b, Schuppe-Koistinen et al. 1994) which is one of the early cellular modifications of proteins. A role of protein S-thiolation/dethiolation as a protective and effective antioxidant function is proposed by

Thomas and Sies (1992), at which protein S-thiolation resulting from a direct reaction of proteins and reduced glutathione with reactive oxygen species (ROS) or a thiol/disulfide exchange reaction, and subsequent dethiolation may protect vulnerable macro-molecules such as proteins, DNA and lipids that can be irreversibly damaged by the ROS. Reactive oxygen species (ROS) generated by the xanthine oxidase reaction with xanthine and reduced glutathione probably proceed through a protein thiyl radical intermediate, that initiates S-thiolation of purified phosphorylase b, creatine kinase (Miller 1990, Park & Thomas 1988), and carbonic anhydrase III (CA III) (Chai et al 1991). The release of O_2^- from human neutrophils as induced by phorbol-12-myristate-13-acetate (PMA) involves S-thiolation of various proteins including actin (Chai 1994b). Phorbol ester-stimulated neutrophils also cause S-thiolation of CA III (Chai 1994a). These S-thiolated proteins return to their original forms by dethiolation or other reduction processes.

Recently Terada et al. (1993) have used ^{14}C-cystine to S-thiolate hGST P1-1 by thiol/disulfide exchange and showed that labeled cystine was incorporated into the protein by 1h. But they did not include thiol/disulfide exchange reaction

between hGST P1-1 and glutathione disulfide (GSSG) which is the most abundant disulfide in cells in their experiments. In their previous experiment, the incubation of hGST P1-1 with GSSG did not extend beyond 2h (Nishihara et al 1991).

In this paper, the characterization of S-thiolation/dethiolation of purified rGST P1-1 using isoelectric focusing (IEF) gel analysis which can directly detect the S-thiolated forms of proteins was performed. Secondly, by using IEF-gel and SDS-PA gel analyses under non-reducing or reducing conditions, and an immunodetection method for rGST P1-1, S-thiolated forms and the formation of a disulfide between subunits of rGST P1-1 in liver extracts containing rGST P1-1 due to induction of altered hepatic foci were identified suggesting that this π class enzyme is under control at the thiol/disulfide level during the process of carcinogenesis. Thirdly, we investigated whether S-thiolated forms occur in a cultured rat hepatocyte model system. Fourthly, we proposed the five different S-thiolated forms of rGST P1-1 based on IEF gel/SDS-PA gel analyses and three dimensional structure of GST P1-1. Also a specific method for purification of rGST P1-1 using preparative IEF based on the property of S-thiolation of

rGST P1-1 can be used to obtain a pure rGST P1-1 band on IEF gel was introduced.

MATERIALS AND METHODS

Materials

N-ethyl maleimide, galactose, dexamethasone, dithiothreitol (DTT), glutathione, glutathione disulfide, phenylmethylsulfonyl fluoride (PMSF), N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (Hepes), carbodiimide[1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulphonate)], benzimidine, leupeptin, the π class of human glutathione *S*-transferase (hGST P1-1) and rat glutathione *S*-transferases (GSTs) were from Sigma Chemical Co. (St. Louis, MO). Bovine type-I collagen (Vitrogen) was obtained from Collagen (Palo Alto, CA), and collagenase (type I) from Worthington Biochemical Co. (Freedhold, NJ). Leibovitz's L-15 media, bovine insulin, human transferrin, bovine albumin fraction V solution (7.5%), penicillin G, and streptomycin sulfate were purchased from Gibco-BRL (Grand Island, NY). Ampholites (pH 5-8, pH 4-6 and pH 3.5-10) and Percoll were obtained from Pharmacia LKB (Piscataway, NJ). Nitrocellulose membrane was from Bio-Rad Lab. (Richmond, CA). Net-Fix for PAG was from Serva Biochem. Inc., Wesbury, NY.

Treatment of Rats with a Modified Solt-Farber Protocol

Male Sprague-Dawley rats (180-200 g of body weight) were used for the Solt-Farber protocol (Solt 1976) with some modification (Figure 1).

Glutathione *S*-transferase (GST) Assays

Enzyme activity was routinely determined using 1-chloro-2,4-dinitrobenzene (CDNB) and GSH as substrate (Habig et al. 1974). Briefly, GST activity was measured in the presence of 1.0 mM GSH with 1.0 mM CDNB as cosubstrate at 25°C and pH 6.5 in 0.1M potassium phosphate buffer. One unit of GST activity is defined as the amount of enzyme catalyzing the conjugation of 1 μmole of CDNB per minute at 25°C.

Protein Determination

Protein concentrations were measured according to the techniques of Lowry *et al.* (1951). But in the experiment of expression of rGST using a modified Solt-Farber protocol, rat liver cytosolic proteins were measured by the Bradford dye-binding method (Bradford 1976). Bovine serum albumin was used as a standard for protein assays.

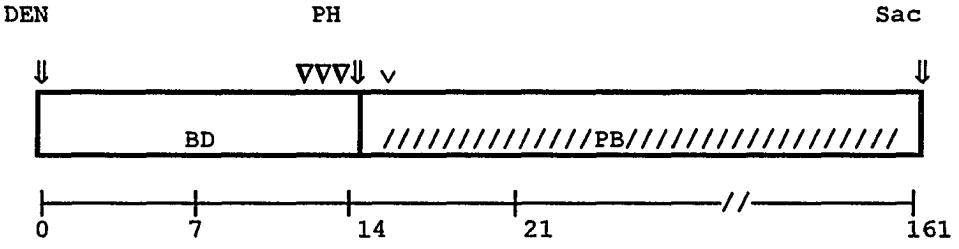


Figure. 1. Schematic representation of a modified Solt-Farber protocol

Basal diet (BD), which contains 40% of kcals from fat and is otherwise based upon the standard AIN-76 rat diet was fed during the first 14 days. Phenobarbital (PB), 0.05 % in basal diet was fed after day 14. A single dose of diethylnitrosamine (DEN, 200 mg/kg body weight) was given by intraperitoneal injection to initiate rats weighing 180-200 g. Daily gavage of 2-acetyl-aminofluorene (2-AAF, ∇, 20 mg/kg body wt.) began on day 11 for 3 consecutive days, followed by partial hepatectomy (PH) on day 14. Three days later a small dose of 2-AAF (v, 5 mg/kg body wt.) was given. Sacrifice (Sac) was performed on day 161.

Purification of the π Class of Rat Glutathione *S*-transferase (rGST P1-1) from Modified Solt-Farber Treated Rat Liver

The π class of rat glutathione *S*-transferase (rGST P1-1) was purified by the method of Satoh, K. *et al.* (1985) with some modification, that is, after each column the fractions were dialyzed with 10 mM DTT overnight to protect sulfhydryl groups of the enzyme. Purified rGST P1-1 was stored at -70°C in small aliquots sufficient for individual tests.

Purification of rGST P1-1 By Preparative Isoelectric Focusing Using Its S-thiolatable Characteristic

The Rotofor cell (Bio-Rad Lab., Hercules, CA), preparative isoelectric focusing apparatus was used to purify rGST P1-1. The preparation of Rat GST P1-1 purification was performed exactly as described above except that the fractions eluted after DEAE-52 anion exchange column were analyzed by immunodot assay (Otata and Cheng 1988) with anti rGST P1-1 antiserum which was prepared with the purified rGST P1-1, and the fractions which had positive response with anti-rGST P1-1 antiserum were loaded into Rotofor cell.

Preparation of Rabbit Polyclonal Antiserum for rGST P1-1

A mixture composed of 0.5 ml of purified rat GST P1-1 (100 μg) and 0.5 ml of Freund's complete adjuvant was

subcutaneously injected at 4 locations on the back of one six month-old male New Zealand rabbit. A booster injection (0.5 ml of a 1:1 mixture, 100 µg of rGST P1-1 and incomplete Freund's adjuvant) followed 2 weeks after the initial injection. Blood was collected every other week from the central ear artery beginning one month after the first injection of the protein. Blood was allowed to clot, and then serum was collected after centrifugation, and was stored frozen in aliquots at -70°C. The antiserum titer was checked by dot immunoassay and 1:1000 gave optimum detection of rGST P1-1 detection.

Hepatocyte isolation and culture

Adult male Sprague-Dolly rats weighing 180-200 g were from Sasco Co. (Omaha, NE), and maintained on wire cages with free access to water and crude cereal-based rat chow. Hepatocytes were isolated by perfusion with 0.05% collagenase as described by Bissell *et al.* (1973) and Bonney *et al.* (1974) with several modification. A rat was anesthetized with sodium pentobarbital (100 mg/kg body weight), and the liver was first perfused at 37°C via the portal vein with 200 ml of perfusion medium, pH 7.5 containing 25 mM Na_2HPO_4, 3 mM KCL, 120 mM NaCl, 11 mM glucose, 0.0005% phenol red, 0.1% BSA (Kilberg, 1983) at 13 ml/min. After 3 to 4 min,

the liver was perfused with 300 ml of the same medium supplemented with 0.05 % Type I collagenase, 4 mM $CaCl_2$, and 5 mg trypsin inhibitor at the same rate. After 15 min of perfusion, the liver was removed from abdomen, washed with phosphate-buffer saline (PBS: 0.2 g KCl:8 g NaCl:0.2 g KH_2PO_4:2.16 g Na_2HPO_4 x $7H_2O$/L, pH 7.4), sieved through a nylon mesh into a flask, and washed with L-15 medium, pH 7.5, supplemented with 18 mM HEPES, 0.2% BSA, 0.05% glucose, and 5 µg/ml insulin. The cell suspension was centrifuged at 200 rpm for 3 min and washed again. The cell pellet was resuspended in a 10% Percoll solution (10 X Hank's buffer plus 90% Percoll solution) and centrifuged at 500 rpm for 10 min to remove dead cells and nonparenchymal cells. Then the pellet containing live cells was resuspended, and washed twice. Cell viability was determined by tryptophan blue exclusion and was greater than 90%. Isolated hepatocytes (0.5×10^6 cells/ml) were plated on 60 mm precoated-tissue culture dishes (Fisher Sci. Co., Chicago, IL) in 5 ml culture medium: Leibovitzs L-15 medium, pH 7.6, supplemented with 18 mM HEPES, 0.2% BSA, 5 µg/ml insulin/transferrin, 1 µM dexamethasone, 5 mg/ml galactose, 5 ng/ml sodium selenite, 100 IU/ml penicillin, and 100 µg/ml streptomycin sulfate. The preparation of the precoated-tissue culture dishes was

done as described by the method of Jauregui et al (1991). The dishes were coated with a bovine type-I collagen preparation (Vitrogen) by covering the surface of the tissue culture dishes with 2 ml of a Vitrogen/carbodiimide solution (100:130 µg/ml) and incubating at 37°C overnight. The collagen solution was removed by aspiration on the following morning and the plates were rinsed twice with PBS and stored at 4°C until ready to use. Dishes were warmed at 37°C for 1 h prior to plating the hepatocytes. Cells were allowed to attach to the dishes for 4 h at 37°C in a humidified incubator with an air atmosphere. Unattached cells were aspirated after 4 h and then the medium containing 25 mM sodium butyrate was added to the cultured hepatocytes, and was then changed once each day. Cells were maintained in culture for 6 days.

Following the incubations, the cells were rinsed twice with cold PBS and harvested by scraping cells in 40 µl cold buffer containing 20 mM β-glycerophosphate, 5 mM EDTA, 5 mM EGTA, 0.1 mM PMSF, 1 mM benzamidine, and 4 µg/ml leupeptin and 50 mM N-ethylmaleimide, pH 7.5. N-ethylmaleimide was prepared freshly in each experiment. Any artifactual modification of sulfhydryls during sample preparation was

avoided by the fast reaction of protein sulfhydryls with N-ethylmaleimide. Cell homogenates were centrifuged at 10,000 x*g* at 4°C for 30 min to obtain a particulate-free cell extract.

Tissue Preparations

At 161 days after treatment, rats were killed by decapitation under CO_2 anesthesia. All liver samples were removed, freeze-clamped in liquid N_2 immediately and made into powdered form, then stored at -70°C until analysis. The powdered form of liver (0.1 g) was homogenized with a sonicator in 0.5 ml of 20 mM β-glycerophosphate (pH 7.5), containing 5 mM EDTA, 5 mM EGTA, 0.1 mM PMSF, 1 mM benzamidine and 4 μg/ml leupeptin. The homogenates were centrifuged at 4°C for 15 min using an air-driven ultracentrifuge with rotor A-100 under 30 psi (Airfuge, Beckman, Palo Alto, CA) to obtain supernatants used for isoelectric focusing gel and SDS-polyacrylamide gel electrophoresis.

Analysis of the π Class Rat Glutathione *S*-transferase Modification

S-thiolation of the π class of rat glutathione *S*-transferase (rGST P1-1) was analyzed by running thin-gel isoelectric focusing as previously described (Thomas &

Beidler 1986). Acrylamide gels (4%) containing 2% LKB ampholytes (1 part of pH 4-6: 5 part of pH 5-8) laid on GelBond PAG film (Pharmacia LKB, Uppsala, Sweden) without and with NetFix backing (Serva Biochem. Inc., Westbury, NY) were prepared for Commassie blue staining or trans-blotting to a nitrocellulose membrane, respectively. Proteins were applied to sample application strips (Serva) on the prefocused gel for 10 min, and were focused for 1h. The focusing was performed at constant voltage, 1500V (2.75 mA/cm and 1.125 W/cm gel width). After running, the gels were either stained with Commassie blue or trans-blotted to a nitrocellulose membrane.

For immuno-blot the gel with NetFix was detached from Gelbond, and equilibrated with cold 0.35% acetic acid for rGST P1-1 for 15 min. Semi-dry transfer equipment (Bio-Rad Lab., Hercules, CA) was used for protein transfer and the transfer was performed at constant voltage, 25 V for 1 h for GST P1-1. Protein on gel with a net positive charge due to protonation by acetic acid was moved to nitrocellulose membrane placed on the cathodic side of the gel. The nitrocellulose membrane was washed four times for 5 min each with *washing medium: 25* mM Tris buffer, pH 7.4 containing 150 mM NaCl, and 0.3% Tween-20. The membrane was incubated in

1% BSA in 25 mM Tris buffer with 150 mM NaCl, pH 7.4 for 30 min at 37°C to block nonspecific binding of antibodies to membranes, and then, was incubated with a 1:500 dilution of a rabbit anti-rGST P1-1 antiserum for 1 h at 37°C as primary antibody. A 1:5000 dilution of anti-rabbit IgG alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO) in 25 mM Tris buffer with 150 mM NaCl, pH 9.1 was used as a secondary antibody, in a 30 min incubation at 37°C. Between incubation steps, the membrane was washed four times for 5 min each with *washing medium*. Finally, protein was stained with an alkaline phosphatase conjugate substrate kit (Bio-Rad Laboratories, Inc. Richmond, CA) at room temperature for 10 min, in which 66 µl 5-bromo-4-chloro-3-indolyphosphate (BCIP) and 33 µl p-nitroblue tetrazolium chloride (NBT) in 10 ml color development solution were used. The reaction was stopped by washing with water, and the membrane was air-dried.

RESULTS

Purification of the π Class of Rat Glutathione S-transferase (rGST P1-1)

As the reported purification of rGST P1-1 by Satoh *et al.* (1985), one major band, which had the same molecular mass as

the π class of human glutathione *S*-transferase (hGST P1-1) on SDS-polyacrylamide gel electrophoresis under reducing conditions, and cross-reactivity with anti-human GST P1-1 antiserum, was observed from the second peak fraction in the elution profile after diethylaminoethyl (DEAE)-52 anion exchange column indicating that the fraction contains rGST P1-1 of high purity (Figure 2A). When this fraction was further analyzed by isoelectric focusing (IEF) electrophoresis, only one band appeared after incubation of the fraction with 20 mM DTT at 30°C for 30 min (Figure 2B). After the preparation of anti-rGST P1-1 antiserum, the purified protein was analyzed with IEF and immuno-blot under the same conditions as shown in lane 1 of Figure 2B. The same band was detected in lane 2 by the antiserum (Figure 2B).

Enzyme-linked antibody detection of rat GST P1-1

The specificity of the rabbit polyclonal antiserum for rat GST P1-1 was tested on an immuno-blot of IEF and SDS- PA gels in Figure 3. Briefly, the cytosolic proteins from livers of modified Solt-Farber protocol treated rats, and rat glutathione *S*-transferases (rGSTs) from Sigma Co. were run on IEF gel (Ampholine pH 3.5-10.0) and SDS-PA gel with

Figure 2. Identification of 24-kDa protein purified from the cytosol of rat liver treated a modified Solt-Farber protocol as the rat glutathione transferase P1-1 (rGST P1-1)

The purification of rat GST P1-1, isoelectrofocusing (IEF) and immuno-blot, and the preparations of polyclonal anti-GST P1-1 antisera were described in MATERIALS AND METHODS. (A). *Lanes 1* and *2,* Coomassie blue stained SDS-PA gel of the human GST P1-1 (hGST P1-1) (2 μg) from Sigma Chemical Co. (St. Louis, MO), and the purified protein (2 μg) under reducing conditions with 20 mM DTT, respectively; *lane 3 and 4,* Immuno-blot of the purified protein and hGST P1-1 (400 ng each), respectively, using anti-human GST P1-1 antiserum. (B). *Lanes 1,* Coomassie blue stained IEF gel (Ampholine pH 4.0-8.0) of the purified protein under reducing conditions; *Lane 2,* Immuno-blot of the purified protein (3 μg) reacted with 20 mM DTT under the same condition of *lane 1* using anti-rGST P1-1 antiserum as a primary antibody. The purified protein was reduced by reacting the protein with 20 mM DTT in 20 mM β-glycerol phosphate buffer, pH 7.5 for 30 minutes and then incubating with 40 mM NEM for 15 minutes. Standard proteins for molecular mass from top to bottom: phophorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (22 kDa), and lysozyme (14 kDa). Standard proteins for pI marker from top to bottom: letil lecitin (pI 8.2, 8.0, 7.8), human hemoglobin C (pI 7.5), equine myoglobin (pI 7.0), equine myoglobin minor band (pI 6.8), human carbonic anhydrase (pI 6.0), β-lactoglobulin (pI 5.1), and phycocyanin (pI 4.8). Standard proteins were stained with Coomassie blue.

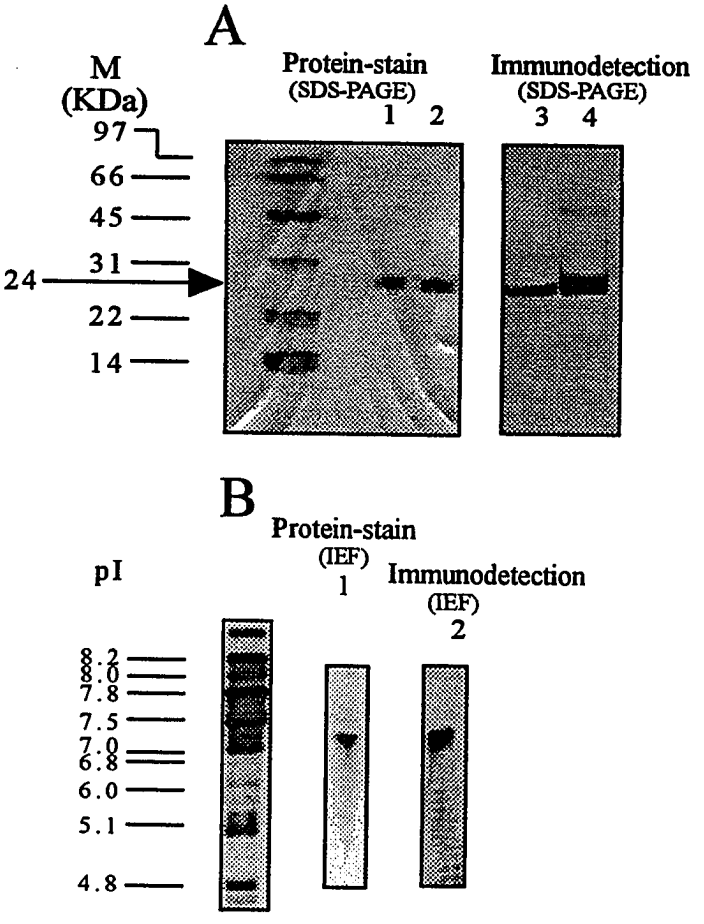
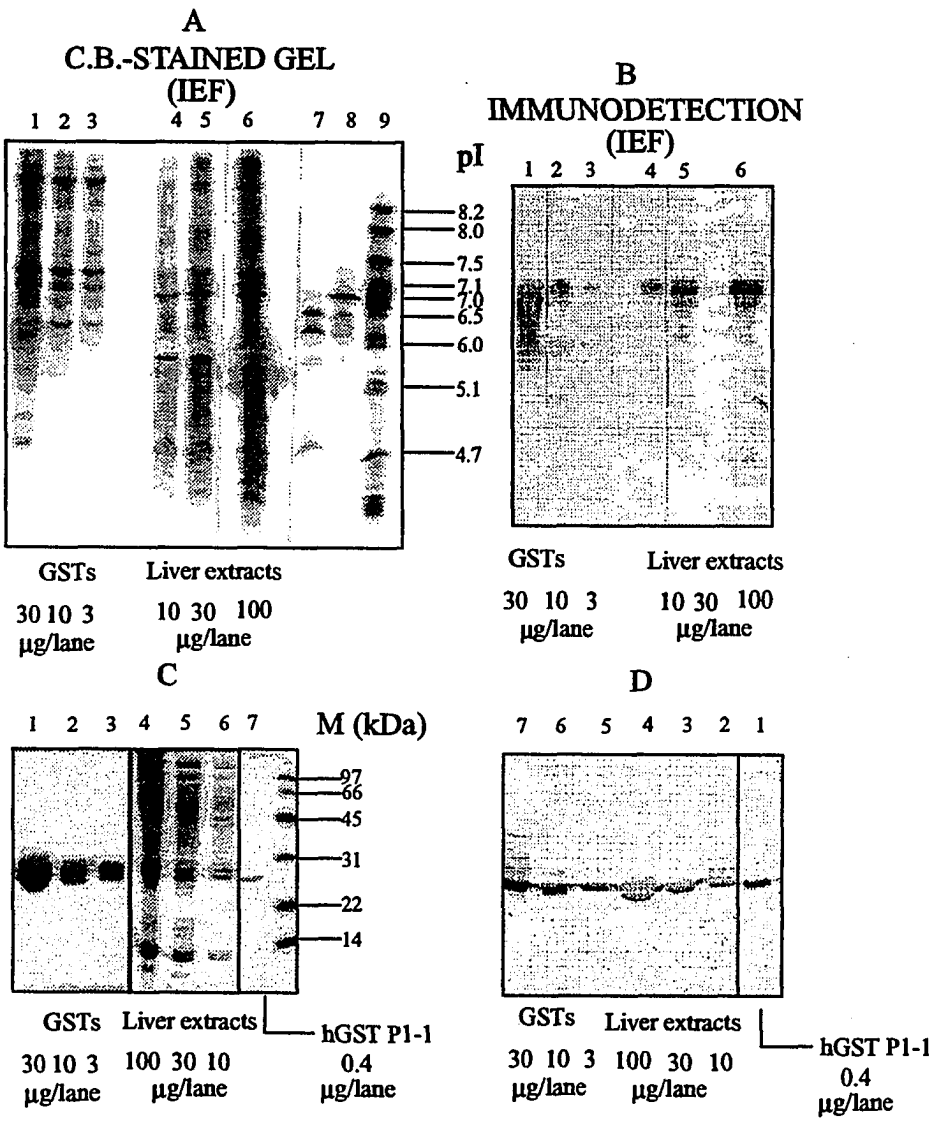

A
M
(KDa)
97
66
45
31
24
22
14
Protein-stain
(SDS-PAGE)
1 2
Immunodetection
(SDS-PAGE)
3 4
B
pI
8.2
8.0
7.8
7.5
7.0
6.8
6.0
5.1
4.8
Protein-stain
(IEF)
1
Immunodetection
(IEF)
2

Figure 3. Specificity of anti-rGST P1-1 antiserum to rGST P1-1 on immuno-blot of both isoelectric focusing (IEF) and SDS-polyacrylamide gels

IEF and immuno-blot were carried out as described in MATERIAL and METHODS. Rat glutathione *S*-transferases (GSTs) was from Sigma Chemical Co. (St. Louis, MO). (A). Coomassie blue stained IEF gel (Ampholine pH 3.5-10.0) of rat glutathione *S*-transferases (GSTs) (*lane 1 to 3*), rat liver extracts treated with a modified Solt-Farber protocol (*lane 4 to 6*), and standards of reduced (*lane 7*)(pI 7.0), partially (pI 6.4) and fully (pI 6.1) S-thiolated carbonic anhydrase III (*lane 8*), and IEF standards (Bio-Rad, Hercules, CA) (*lane 9*). (B). *Lane 1 to 3 and lane 4 to 6*. Immuno-blot using the same samples under condition used in part A using anti-rGST P1-1 antiserum as a primary antibody. (C). The Coomassie blue stained SDS-PA gel of rat GSTs (*lane 1 to 3*), rat liver extracts treated with a modified Solt-Farber protocol (*lane 4 to 6*), and the purified rat GST P1-1 (lane 7) under reducing conditions. (D). *Lane 1 to 3, lane 4 to 6, and lane 7*. Immuno-blot using the same samples under reducing conditions used in part C using anti-rGST P1-1 antibody as a primary antibody. MW and pI, same standard proteins as used in Figure 2.


A
C.B.-STAINED GEL
(IEF)
1 2 3 4 5 6 7 8 9
pI
8.2
8.0
7.5
7.1
7.0
6.5
6.0
5.1
4.7
GSTs
30 10 3
µg/lane
Liver extracts
10 30 100
µg/lane
B
IMMUNODETECTION
(IEF)
1 2 3 4 5 6
GSTs
30 10 3
µg/lane
Liver extracts
10 30 100
µg/lane
C
1 2 3 4 5 6 7
M (kDa)
97
66
45
31
22
14
GSTs
30 10 3
µg/lane
Liver extracts
100 30 10
µg/lane
hGST P1-1
0.4
µg/lane
D
7 6 5 4 3 2 1
GSTs
30 10 3
µg/lane
Liver extracts
100 30 10
µg/lane
hGST P1-1
0.4
µg/lane

20 mM DTT, and then stained with Coomassie blue staining or immunoblotted with anti-rGST P1-1 antiserum as a primary antibody. Figure 3A shows the pIs of rGSTs (lane 1 to 3) and a modified Solt-Farber treated liver extract (lane 4 and 6). Figure 3B shows the immunoreactivity of these proteins with anti-rGST P1-1 antiserum. The modified Solt-Farber treated liver extract (lane 4 to 6) in Figure 3B was detected by anti-rGST P1-1 antiserum which gave one major band at pI 7.1 as rGSTs did (lane 1 to 3) in Figure 3B.

The antiserum specificity was also tested with a denaturing system such as a SDS-PA gel which is shown in Figure 3C and 3D, in which the antiserum reacted with rGST P-1 (lane 1 to 3) and the liver extract (lane 4 to 6) in Figure 3D. Purified rGST P1-1 (400 ng) was easily immunodetected by using the antiserum. This rabbit polyclonal antiserum against rGST p1-1 was used to characterize the fraction which was purified with preparative isoelectric focusing for another rGST P1-1 purification.

Preparative Isoelectric Focusing for rGST P1-1 Purification Using Its S-thiolatable Characteristic

When we have prepared another sample of rGST P1-1, the second peak fraction in the elution profile after DEAE-52 column gave a positive response in immuno-dot assay using

anti rGST P1-1 antiserum (data not shown), but showed impurity of the protein bands based on the analysis by SDS- and IEF- gel electrophoresis in Figure 4. The pooled fractions (fraction No. 43-48) of the second peak after DEAE-52 anionic column elution gave two major bands on SDS-PA gel staining under reducing conditions (Figure 4A) compared with one band from the first enzyme preparation (Figure 2A). And on IEF gel staining, each fractions showed several similar bands (Figure 4B), and among these fractions, fraction No. 45 (lane 1 and 2, Figure 4C) was analyzed to see whether it was thiolatable with diamide, in which one band migrated at a similar p*I* of band (e) in Figure 7 with diamide reaction and several bands moved with DTT treatment.

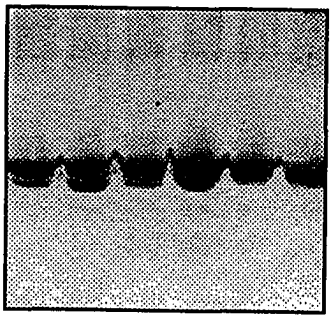
Thus we decided to use a preparative isoelectric focusing apparatus, the Rotofor cell (Bio-Rad Lab., Hercules, CA) to purify further rGST P1-1 using its S-thiolatable characteristic. Fraction #44-46 (Figure 4A and B) were pooled, reduced by incubating in 20 mM β-glycerophosphate, pH 7.5 containing 10 mM DTT for 1h at 30°C, and dialyzed against 20 mM β-glycerophosphate, pH 7.5 overnight. The pooled sample was incubated with 5 mM GSH for 10 min followed by reaction with 6 mM diamide for 5 min in 20 mM β-glycero-

Figure 4. The analysis of the second preparation for rat GST P1-1 purification with fractions concentrated after diethylaminoethyl (DEAE)-52 anionic column elution

The purification of rGST P1-1 using preparative IEF was described under MATERIALS AND METHOD. (A). Coomassie blue stained SDS-PA gel of fractions concentrated after diethylaminoethyl (DEAE)-52 anionic column elution (*Fraction No.* 43-47) under reducing conditions. (B). Coomassie blue stained IEF gel with the same fractions as those in part A. (C). Coomassie blue stained IEF gel of fraction No. 45 which was incubated with 5 mM reduced glutathione for 10 min and then reacted with 6 mM diamide for 5 min in 20 mM β-glycerophosphate buffer at pH 7.5.at 30°C (*lane 1*). *Lanes 2* shows that the reaction was also reduced with 20 mM dithiothreitol before IEF.

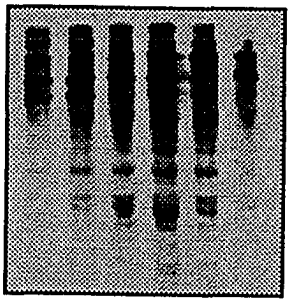
A

Fraction No. 43 44 45 46 47 48



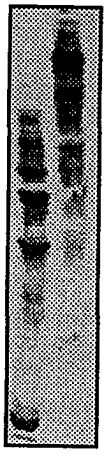
B

Fraction No. 43 44 45 46 47 48



C

45



1 2

phosphate, pH 7.5 at 30°C, and then was diluted with water containing 2% LKB ampholytes (1 part of pH 4-6: 5 part of pH 5-8) to make a total volume of 40 ml, and applied the Rotofor cell. The focusing was performed at constant power, 12 W for 4 h with ice cooling, and fractions #1-#20 (≅ 2 ml/fraction) were collected.

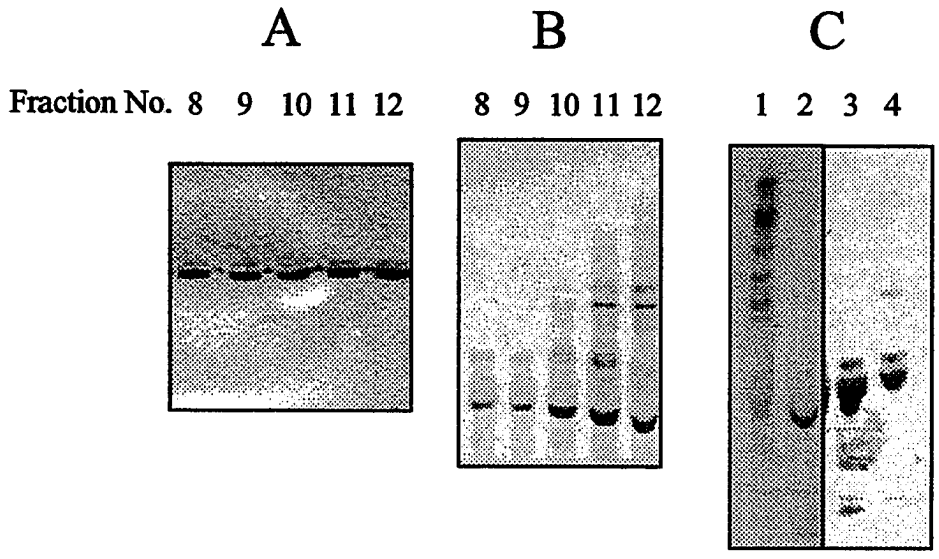
Characterization of the Pooled Sample from the Rotofor by Immuno-blot Analysis Using Anti rGST P1-1 antiserum

After using the Rotofor cell to separate the S-thiolatable band, the fractions which gave positive responses in immunodot assay with anti-rGST P1-1 antiserum were analyzed with SDS-PAGE and isoelectric focusing (fraction No. 8-12) in Figure 5A and 5B. Although these fractions gave one major band and one minor band (fraction No. lane 8-10) in Figure 5A on SDS-PAGE under reducing condition, fraction No. 8-10 showed a similar band pattern with one major band having high purity on IEF gel staining (fraction No. 8-10) in Figure 5B. These fractions were pooled and this pooled sample was analyzed further by IEF. Figure 5C shows the divided gels run at the same time, in which one is for Commassie blue stain (lane 1 and 2) and the other is for immuno-blot (lane 3 and 4). One major band was detected from the fraction before loading the Rotofor cell (lane 3) and the pooled

Figure 5. Preparative isoelectric focusing for rGST P1-1 purification using its S-thiolatable characteristic

The pooled fraction (No. 44-46) was reduced by 10 mM dithiothretol (DTT) in 20 mM in 20 mM β-glycerophosphate buffer at pH 7.5.at 30°C for 1h, and dialyzed against 20 mM β-glycerophosphate at pH 7.5 overnight. This fraction was used for preparative IEF for rGST P1-1 purification.

The preparative IEF using the Rotofor cell (Bio-Rad Laboratory, Hercules, CA) was described under MATERIALS METHOD. (A) and (B). Coomassie blue stained SDS-PA gel and IEF gel of fraction after separation using Rotofor cell, respectively. (C). *Lane 1* and *2*, Coomassie blue stained IEF gel of the fraction before loading Rotofor cell and the pooled fraction (No. 8-10) after separation; *Lane 3* and *4*, Immuno blotting of the same samples in *lane 1* and *2*.


A
B
C
Fraction No. 8 9 10 11 12
8 9 10 11 12
1 2 3 4

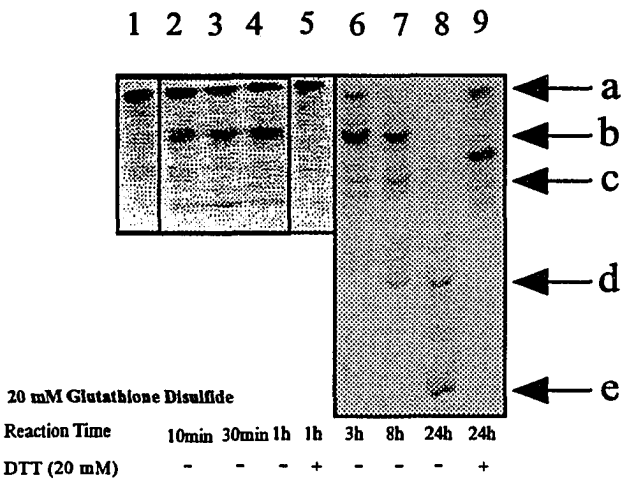
fraction (lane 4) (fraction No. 8-10) after Rotofor separation.

S-thiolation of the π Class of Rat Glutathione *S*-transferase by Thiol/disulfide Exchange with GSSG

The reaction of the π class of rat glutathione *S*-transferase (rGST P1-1) with GSSG was analyzed by IEF. If a protein were S-thiolated by GSSG or glutathiolated through thiol/disulfide exchange, the p*I* of the protein would be altered because the addition of glutathione (GSH) to a protein theoretically changes the net charge of the protein by -1 (the sum of total charges of $-NH_3^+$ and $-COO^-$ from glutamate and $-COO^-$ from glycine residue of GSH). Thus, an S-thiolated or glutathiolated protein produced by thiol/disulfide exchange with GSSG and then alkylated with N-ethylmaleimide(NEM) after S-thiolation would migrate and focus with more acidic p*I* than the reduced form of the protein.

The time course of *S*-thiolation of rGSTP1-1 by incubating the protein with 20 mM GSSG at 30°C is shown in Figure 6. The S-thiolation of rGST P1-1 during 10 min, 30 min and 1h incubation (lanes 2-4) showed similar migration pattern of bands indicating that band (b) was derived from band (a), but

Figure 6. S-thiolation of the π class of rat glutathione *S*-transferase (rGST P1-1) by glutathione disulfide (GSSG) Commassie Blue stained IEF gel of the reduced rat GST P1-1 (*lane 1*)and rat GST P1-1 reacted with 20 mM glutathione disulfide (GSSG) at 30°C for the indicated time (*lane 2 to 4 and lane 6 to 8*). Each reaction contained 5μg of the protein in 20 mM β-glycerophosphate buffer at pH 7.5. After incubation with GSSG, the protein was alkylated with 40 mM N-ethyl maleimide (NEM) for 15 min at the room temperature. *Lanes 5* and *9* shows that the reaction was also reduced with 20 mM dithiothreitol (DTT) at 30°C for 30 min before IEF.


1 2 3 4 5 6 7 8 9
a
b
c
d
e
20 mM Glutathione Disulfide
Reaction Time 10min 30min 1h 1h 3h 8h 24h 24h
DTT (20 mM) - - - + - - - +

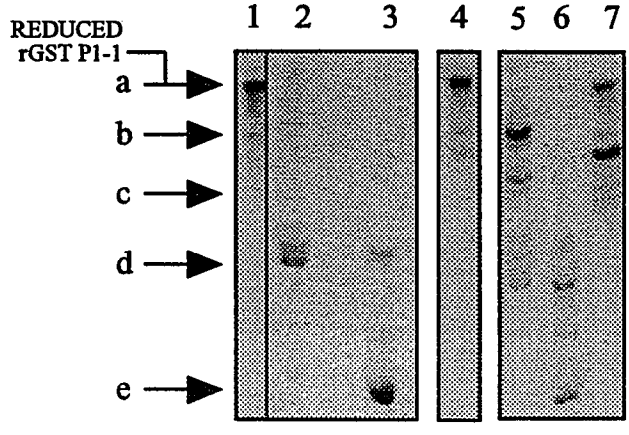
band (a) still remained. Lane 5 showed that the reaction was fully reversible by dithiothretol (DTT) within 1h of incubation with GSSG. The reduced rGST p1-1 band (a) still remained after S-thiolation of rGST P1-1 with 20 mM GSSG for 3h incubation. The time course for S-thiolation of rGST P1-1 with 20 mM GSSG was extended from 3h up to 24h in lanes 6-9. The time sequence of S-thiolation of rGST P1-1 suggests that band (c) (lane 6 and 7) is derived from band (b), that band (d) (lane 8) is from band (c), and that band (e) (lane 8) is from band (d). Lane 9 showed that S-thiolated bands were not fully reduced to the original band (a) by DTT after 24h incubation with GSSG.

S-thiolation of rGST P1-1 by Diamide

Because an adequate glutathione pool protects carbonic anhydrase III from irreversible oxidation by formation of the S-thiolated protein (Lii et al. 1994), reduced glutathione(GSH) was added to a reaction system using diamide to induce S-thiolation. The protein was incubated with 5 mM GSH for 10 min and then, reacted with 4 mM or 6 mM diamide for 5 min in 20 mM β-glycerophosphate, pH 7.5 at 30°C. The effect of diamide on the S-thiolation of rGST P1-1 is shown in Figure 7. The reaction with the lower concentration of diamide (4 mM) (lane 2) showed thiol-modified protein

Figure 7. Comparison of S-thiolation of the π class of rat glutathione *S*-transferase (rGST P1-1) by diamide and glutathione disulfide

Commassie Blue stained IEF gel. For S-thiolation of rat GST P1-1 with diamide, the enzyme was first incubated with 5 mM GSH for 10 min and then treated with diamide at the indicated concentration (*lanes 2, 3,* and 4) for 5 min in 20 mM β-glycerophosphate buffer at pH 7.5.at 30°C. Each reaction contained 5μg of the protein. For S-thiolation of rat GST P1-1 with glutathione disulfide (GSSG), the enzyme was treated with 20 mM GSSG during the indicated times (*lanes 5, 6* and 7) in 20 mM β-glycerophosphate buffer at pH 7.5.at 30°C. After incubation with GSSG or diamide, the protein was alkylated with 40 mM NEM for 15 min at the room temperature. *Lanes 4* and 7 show that the reaction was also reduced with 20 mM dithiothreitol before IEF.



Reaction Times	5min	5min	5min	8h	24h	24h
	Diamide (4mM)	(6mM)	(6mM)	GSSG	(20mM)	
DTT (20 mM)	-	-	+	-	-	+
GSH (5 mM)	+	+	+	-	-	-

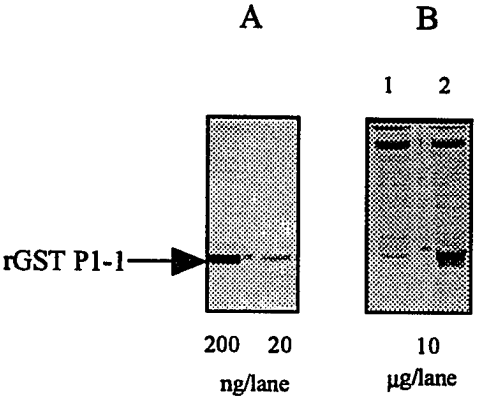
focusing at similar p*I* to band (d) (lane 5), thiol-modified form of rGST P1-1 with GSSG, whereas the thiol-modified protein with 6 mM diamide (lane 3) moved further at the p*I* of band (e) of lane 6. Based on the S-thiolation pattern of rGST P1-1 on IEF, the reaction of the protein with 20 mM GSSG for 8h and 24h gave similar results of S-thiolation to those of S-thiolation with 4 mM and 6 mM diamide for 5 min, respectively. The band (d) in lane 2 and (e) in lane 3 were returned to the reduced form after 20 mM DTT treatment in Figure 7.

S-thiolation of Rat GST P1-1 from the Modified Solt-Farber Treated Cytosolic Fraction by Diamide

Because rGST P1-1 is present in very small amounts in normal rat liver whereas it is significantly induced in liver bearing altered hepatic foci (Kitahara et al. 1984), we have used a modified Solt-Farber protocol (Figure 1) to induce rGST P1-1 in rat liver. The amount of rGST P1-1 was quantified by scanning of immuno-blot, using anti-rGST P1-1 antiserum and the protein was quantified by comparison with known amounts of rGST P1-1. As seen in Figure 8, rGST P1-1 in rat liver treated with a modified Solt-Farber protocol showed a ten fold induction in rGST P1-1 content compared with the controls. Using this liver extract having induced

Figure 8. Expression of rat GST P1-1 (rGST P1-1) from the experiment using a modified Solt-Farber protocol.

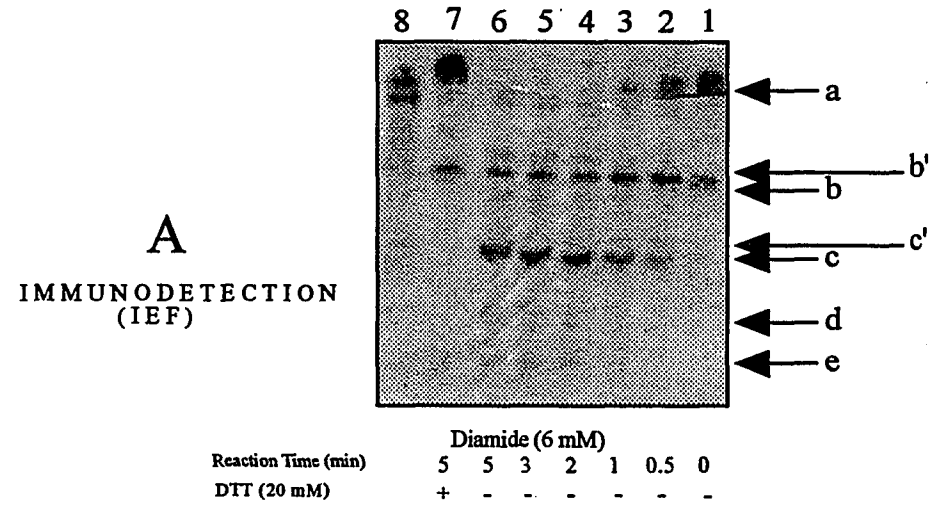
(A). Immuno blot of rat GST P1-1 for standards (200 ng, 10 ng). The preparation of rat liver cytosolic fraction was described under **MATERIALS AND METHODS**. (B). *Lanes 1* and (10 µg), Immuno blot of rat liver cytosolic fraction from control; *Lanes 2* (10 µg), Immuno blot of rat liver cytosolic fraction from a modified Solt-Farber protocol.

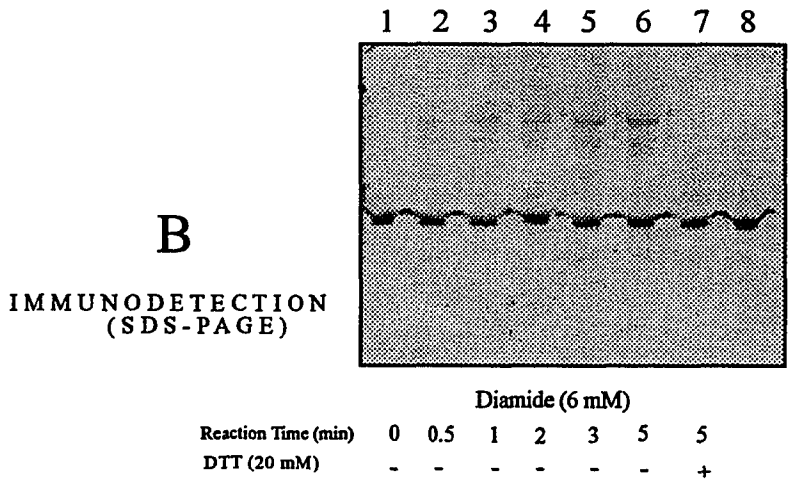

A
B
1
2
rGST P1-1
200
20
ng/lane
10
µg/lane

rGST P1-1, an S-thiolation experiment by diamide was performed (Figure 9). The time sequence of S-thiolation of rGST P1-1 by diamide suggests that band (b) comes from band (a) and band (c) from band (b) (Figure 9A). Also band (d) and (e) appeared slightly with diamide treatment. Interestingly, two minor bands (band (b') and (c'))appeared after 1 min reaction with diamide (lane 3), and these bands disappeared with DTT treatment (Figure 9A). Because diamide, a thiol-oxidizing agent, can react with thiols including glutathione and protein containing sulfhydryl groups (Kosower et al. 1972), dimeric forms of rGST P1-1, in which the subunits are connected by disulfide bond, can be formed. When a dimer is formed, it should be separable from monomeric subunit using SDS-PA gel without reducing agents such as DTT and β-mercapoethanol. The same samples used in Figure 9A were separated on an SDS-PA gel under non-reducing conditions, and then transferred to a nitrocellulose membrane to detect rGST P1-1 immunochemically (Figure 9B). As shown in Figure 9B, on SDS-PA gel higher molecular weight bands compared to the original band appeared after 0.5 min reaction with diamide and these bands disappeared with reduction by DTT.

Figure 9. S-thiolation and protein-protein disulfide formation of rat GST P1-1 (rGST P1-1) from rat liver cytosolic fraction by diamide

For S-thiolation of rGST P1-1 from rat liver treated with a modified Solt-Farber protocol with diamide, liver cytosolic fraction containing 5 mM GSH was prepared as described under MATERIALS AND METHODS. The cytosolic fraction was reacted with 6 mM diamide for 0, 0.5, 1, 2, 3 and 5 min (*lane 1 to* 7) in 20 mM β-glycerophosphate buffer pH 7.5.at 30°C. *Lane 8.* The cytosolic fraction before reacting with diamide. The cytosolic proteins from diamide-treated rat liver were first separated on an IEF gel/SDS-PA gel and then transferred to a nitrocellulose membrane and detected immunochemically using anti-rGST P1-1 antiserum as a primary antibody. 20 mM *N*-ethyl maleimide (NEM) was added just before use in order to block reactions of protein sulfhydryls and other thiols during preparation. (A). Immuno-bolt of IEF gel. (B) Immuno-blot of SDS-PA gel using the same sample in part (A).


A
IMMUNODETECTION
(IEF)
8 7 6 5 4 3 2 1
a
b'
b
c'
c
d
e
Diamide (6 mM)
Reaction Time (min) 5 5 3 2 1 0.5 0
DTT (20 mM) + - - - - - -


B
IMMUNODETECTION
(SDS-PAGE)
1 2 3 4 5 6 7 8
Diamide (6 mM)
Reaction Time (min) 0 0.5 1 2 3 5 5
DTT (20 mM) - - - - - - +

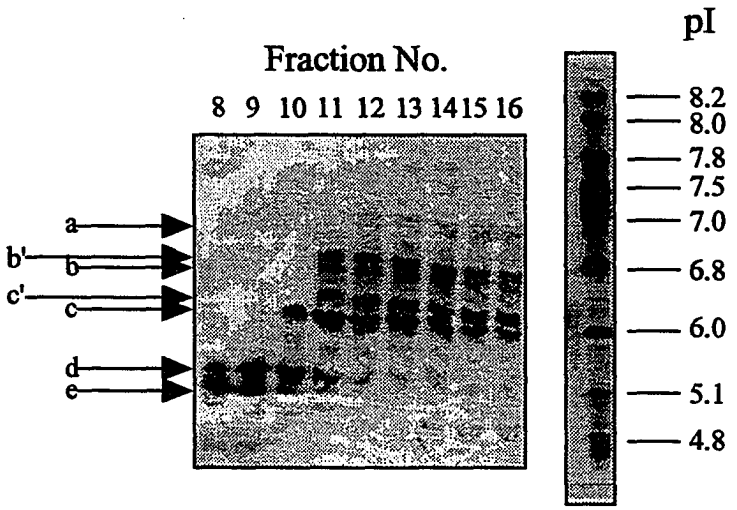
Identification of Protein-protein Disulfide Bonds of rGST P1-1 by IEF and SDS-PA Gel Analysis

To identify the bands due to protein-protein disulfide formation, we reacted liver extract containing induced rGST P1-1 by a modified Solt-Farber protocol with 6 mM diamide, then loaded it on Rotofor to separate the thiolated forms of rGST by pI, and analyzed fractions containing different thiolated forms of rGST P1-1 on SDS-PA gel under non- or reducing conditions, and IEF gel (see Figure 10 legend for detail). Fraction No. 8 to 16 showed several bands which were detected with anti-rGST P1-1 antiserum (Figure 10A). As shown in Figure 9A, five major bands, which have similar pIs with band (a) to (e) were found, and two bands which appear to be band (b') and band (c') in Figure 9A were also found (Figure 10A). The fractions containing different thiolated forms of rGST P1-1 (fraction No. 9, 10, 11, 14 and 15) were analyzed on SDS-PA gel under non- or reducing conditions (Figure 10B). Under non-reducing conditions, five bands (band (A) to (E)) were found. Band (A) (50 kD) indicating that it is dimer of rGST P1-1 (24 kD), and the fraction No. 8 to 16 analyzed contain dimer form of GST P1-1 was found in fraction No. 8 to 16. In addition fraction No. 9 and 10 did not have band (C) and (D) compared

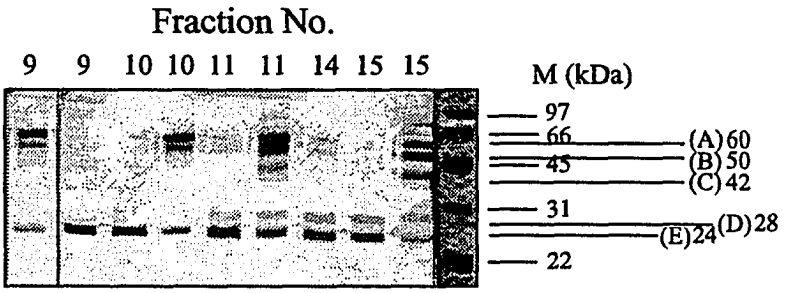
Figure 10. Identification of protein-protein disulfide form of rat GST P1-1 of rat liver cytosolic fraction by diamide using Rotofor.

For S-thiolation of rGST P1-1 from rat liver treated with a modified Solt-Farber protocol with diamide, liver cytosolic fraction containing 5 mM GSH was prepared as described under MATERIALS AND METHODS, and in this experiment the powdered form of liver (2 g) was homogenized in 18 ml of 20 mM β-glycerophosphate (pH 7.5). The cytosolic fraction was obtained by ultracentrifuging 100,000 x g at 4°C for 1 h two times. The supernatent was reduced with 20 mM DTT at 30°C for 30 min and dialyzed against 20 mM β-glycerophosphate buffer (pH 7.5) overnight replacing the buffer every 4 h. The dialyzed sample was incubated with 5 mM GSH for 10min, then reacted with 6 mM diamide for 5 min, alkylated with 20 mM NEM, and then was mixed with same volume of water containing 2% LKB ampholytes (pH 3.5-10.0) to make a volume of 17 ml, was loaded on the Rotofor cell, and was collected as 20 fractions. The fraction No. 8 to 16 which gave anti rGST P1-1 positive response were separated on an IEF gel/SDS-PA gel and then transferred to a nitrocellulose membrane and detected immunochemically using anti-rGST P1-1 antiserum as a primary antibody. (A). Immuno-bolt of IEF gel of the active fractions. (B) Immuno-blot of SDS-PA gel using the specified fractions under reducing and non-reducing conditions. M and pI, same standard proteins as used in Figure 2.

A


pI
Fraction No.
8 9 10 11 12 13 14 15 16
a
b'
b
c'
c
d
e
8.2
8.0
7.8
7.5
7.0
6.8
6.0
5.1
4.8

B


Fraction No.
9 9 10 10 11 11 14 15 15
M (kDa)
97
66
45
31
22
(A)60
(B)50
(C)42
(E)24
(D)28

DTT (20 mM) - + + - + - + + -

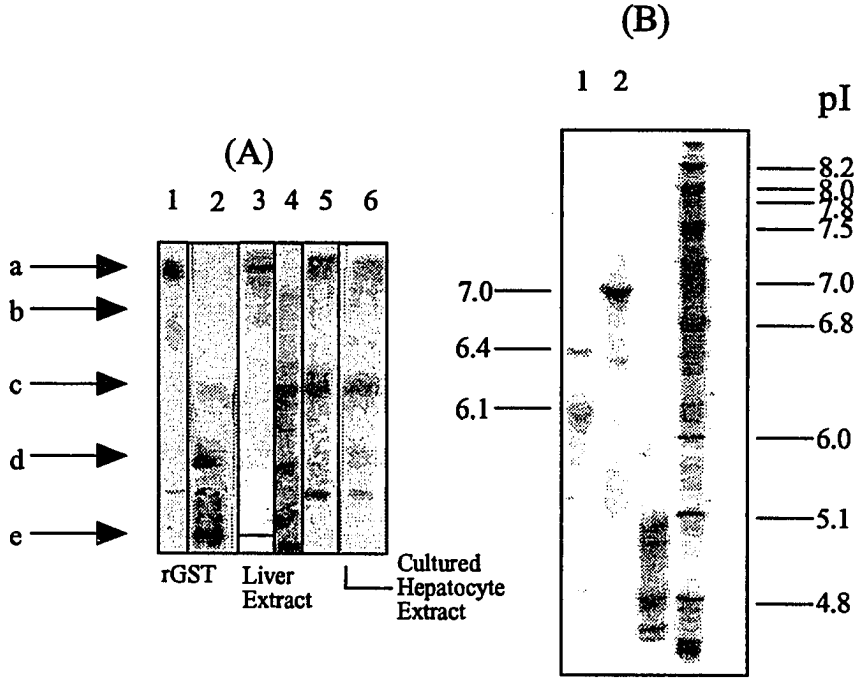
with fraction No. 11 and 15. Under reducing conditions, fraction No. 9 and 10 gave band (E)(24 kD), whereas fraction No. 11, 14 and 15 had one extra band (D)(28 kD) which was not reduced by DTT.

Comparison of Thiolated Band Forms of Purified rGST P1-1, a Modified Solt-Farber treated Liver Extract and Cultured Hepatocyte Extract from Male Rat on IEF Gel Followed by Immuno-blot Using Anti rGST P1-1 Antiserum

The reduced purified rGST P1-1 (lane 1) showed band (a); pI 7.1, and when it is thiolated with 6 mM diamide for 5 min it gave band (d), pI 5.5 and band (e), pI 4.9 (lane 2) (Figure 11A). Liver extract containing induced rGST P1-1 treated with a modified Solt-Farber protocol gave band (a) and band (b); pI 6.9 (lane 3). When this liver extract was reacted with 6 mM diamide for 5 min, it showed band (c); pI 6.2, band (d), and band (e) (lane 4), . The reduction of liver extract treated with diamide with 20 mM DTT for 30 min gave band (a) and band (c) (lane 5). It is worthwhile to examine whether these thiolated forms of rGST P1-1 occur in rat hepatocytes. Because normal rat hepatocytes do not express rGST significantly and control cultured hepatocytes for 6 days contain less rGST P1-1 (data not shown), we cultured the hepatocyte in media containing 2.5 mM sodium

Figure 11. Comarison of band patterns of rat GST P1-1, a Modified Solt-Farber protocol treated rat liver extract and cultured hepatocyte extract on IEF gel.

(A) Immuno-blot of rGST P1-1 reduced with 20 mM DTT at 30°C for 30 min after incubation with 6 mM diamde for 5 min (*lane 1*), rGST P1-1 incubated with 6 mM diamde for 5 min (*lane 2*); 3 µg/lane, liver cytosolic fraction from a modified Solt-Farber protocol treated rat reduced with 20 mM DTT at 30°C for 30 min after incubation with 6 mM diamde for 5 min (*lane 3*), the liver cytosolic fraction incubated with 6 mM diamde for 5 min (*lane 4*); 30 µg/lane, and rat cultured hepatocyte treated with 2.5 mM sodium butyrate for 6 days (*lane 5*); 90µg/lane. (B) standards of partially (pI 6.4), fully (pI 6.1) S-thiolated (*lane 1*), and carbonic anhydrase III reduced (*lane 2*)(pI 7.0). pI, same standard proteins as used in Figure 2.


(A)
1 2 3 4 5 6
a
b
c
d
e
rGST
Liver Extract
Cultured Hepatocyte Extract
(B)
1 2
pI
8.2
8.0
7.8
7.5
7.0
6.8
6.0
5.1
4.8
7.0
6.4
6.1

butyrate for 6 days to induce rGST P1-1. The cultured hepatocyte extract treated with 2.5 mM sodium butyrate for 6 days showed band (a), (c), and (d) (lane 6).

Estimated Distance to Evaluate Intrasubunit or Intersubunit Disulfide Bonds between Cysteine Residues Based on a Data Base Containing the Three-dimensional Structure of Pig GST P1-1

Base on protein data base containing the three-dimensional structure of pGST P1-1 (Figure 12), the intrasubunit distances of cysteine residues are the following; A14-A99: 19Å, A45-A99: 19Å, A14-A167: 20Å, A45-A167: 32Å and A14-A45: 33Å in which A represents the same subunit, and intersubunit distance of cysteine residues are the following; A45-B45: 38Å and A99-B99: 4.8Å in which B represent the different subunit.

DISCUSSION

Based on a data base containing the three-dimensional structure of the class π glutathione *S*-transferase from pig lung (Dirr et al. 1994), we could estimated the locations of reactive cysteines to determine which residues would be most likely to form disulfides (Figure 12). Disulfide formation between subunits by the reaction of rGST P1-1 in rat liver extract with diamide was confirmed (Figure 9). These

Figure 12. Stereoview of the interface between the two π class of glutathione *S*-transferase (pGST P1-1) subunit

This three-dimensional structure of pGST P1-1 bound with glutathione sulfonate was obtained from protein data bank. The Cys 45 residue is located outer surface of the subunit, and at or near catalytic Tyr 7 residue among other cysteine residues.


DH A7
S A45
S A49
S B99
B209
DH A7
S A45
S A49
S B99
B209

protein-protein disulfides of rGST P1-1 by reaction with diamide were identified by running, and separating thiolated forms with different pIs using a Rotofor cell, and analyzing IEF gel and SDS-PA gel under non- or reducing conditions (Figure 10). The band (B) in Figure 10B, which is a protein-protein dimer with a disulfide bond was identified because this band has twice the molecular mass (50 kD) of rGST P1-1's subunit (24 kD), and can be reduced by DTT treatment giving band (E); 24 kD. As shown in Figure 10A, this band (B) occurs in fraction No. 8 to 16 suggesting that the fractions containing band (d) and (e) have a protein-protein disulfide form, and other fractions containing band (b) and (c) have another disulfide form of rGST P1-1. In Figure 9A, lane 1 has no band (c), and did not show dimer formation on SDS-PA gel under non- reducing conditions suggesting band (c) instead of band (b) is a disulfide form of rGST P1-1. In this experiment, it is not known which band from band (d) and band (e) is responsible for another disulfide form in Figure 11. The characterization of band (A), (C) and (D) in Figure 10B is not possible in this experiment, but band (C) and (D) appeared in fraction No. 11 and 15 in Figure 10B which gave extra band (b') and (c') in Figure 10(A) indicating that band (C) and (D) on SDS-PA gel

under non-reducing conditions seem to be responsible for band (b') and (c') on IEF gel in Figure 10. The three-dimensional structure of pGST P1-1 indicates that A99-B99 (4.8Å) corresponding to A101-B101 in rat GST p1-1, distance between subunits is within the range permitting formation of a disulfide bond, the diamide reaction with sulfhydryls of rGST P1-1 is likely to produce disulfide formation between Cys 101 residue of rGST P1-1' each subunit by diamide reaction. Shen *et al.*(1993) proposed that the formation of an intersubunit disulfide bond between Cys 47 and Cys 47 occurs by independently substituting cysteine residues of rGST P1-1 with alanine residues (C14A, C47A, C101A, and C169A) and reacting with H_2O_2. Because of the distance Cys 47-Cys 47 (38Å) on each subunit and the opposite location of Cys 47 on each subunit, a disulfide bond between Cys 47 and Cys 47 is not feasible. When the restrictly site mutated-rGST P1-1s are treated with H_2O_2, and are analyzed with IEF gel and SDS-PA gel under non-reducing or reducing conditions, it will give more specific information on disulfide formation between subunits. Also the experiment of S-thiolation of mouse GST P1-1 with diamide in which this enzyme lacks Cys 101 on the subunit and can not

make intersubunit disulfide bond according to our proposed thiolated forms of rGST P1-1 will be interesting.

Based on our data and the three-dimensional structure of pGST P1-1, we proposed the five possible S-thiolated forms of rGST P1-1. When rGST P1-1 is glutathiolated through SH/SS change, the pI of the enzyme would be altered because of the addition of GSH resulting in a change of net charge of the enzyme by -1. As shown in Figure 13, band (a) represents the reduced form of rGST P1-1. Because this protein has two reactive sulfhydryls, Cys 47 and Cys 101 on each subunit, and Cys 47 seems to be more reactive than Cys 101 in term of sulfhydryl modification, the next S-thiolated form is mono-glutathiolated form of either subunit: band (b) at Cys 47. As the S-thiolation continues, disulfide formation between Cys 101 and Cys 101 from each subunit having a mono-glutathiolated form begins to appear leading to mono-glutathiolated disulfide form: band (c). On the other hand glutathiolated form of both subunit at Cys 47 has further move toward basic pI on IEF because of more negative net charge of the protein which lacks of intersubunit disulfide bond leading to di-glutathiolated form: band (d). Final S-thiolated form of rGST P1-1 is di- glutathiolated disulfide form which has intersubunit disulfide bond: band (e).

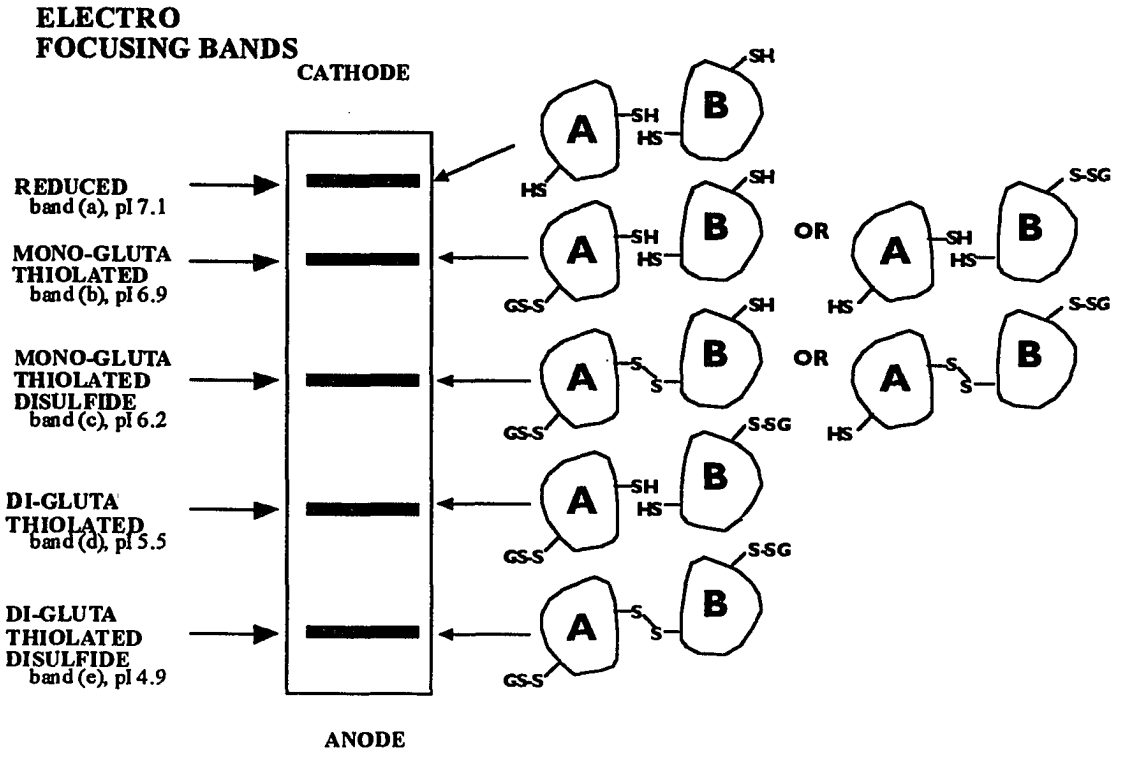


Figure 13. A Proposed model for S-thiolation of rGST P1-1.

In this model, A and B represent each subunit of rGST P1-1. Protein-S-SG is the mixed disulfide with glutathione.

Nishihara *et al.* (1991) have used ^{14}C-cystine instead of GSSG to S-thiolate hGST P1-1 by thiol/disulfide exchange and showed that labeled cystine was incorporated into the protein by 2h. Figure 3 shows S-thiolation of rGST P1-1 by thiol/disulfide exchange with GSSG, and indicates that this reaction occurs at a slow rate. The reaction of rGST P1-1 with GSSG for 1 or 3h gave only partially S-thiolated bands.

The experiments described here show that the π class of rat glutathione *S*-transferase (rGST P1-1) can be thiolated *in vitro* using purified rGST P1-1, and liver extract and cultured rat hepatocyte extract containing rGST P1-1 by using isoelectric focusing (IEF) method which can separate S-thiolated proteins according to their net charge. In IEF/immunodetection methods used in our study, 0.35% acetic acid solution was used to transfer rGST P1-1 to nitrocellulose membranes. This concentration of acetic acid solution for transfer of the fraction was used instead of using 0.7% acetic acid solution which is used for transferring CA III, and gave more efficient transfer of the fraction than higher concentration of acetic acid solution (data not shown). This modification may contribute to increased detection capacity for S-thiolation/dethiolation of

π class enzymes *in vivo*.

In this paper, the characterization by isoelectric focusing (IEF) gel/SDS-PA gel electrophoresis and immuno-blot methods of S-thiolation/dethiolation and protein-protein disulfide bond formation of the π class of rat glutathione *S*-transferase (rGST P1-1) was performed with 1) purified rGST P1-1; 2) male Sprague-Dawley rat liver extract containing large amounts of rGST P1-1, as induced by a modified Solt-Farber protocol; 3) cultured rat hepatocyte extract containing induced rGST P1-1 by 2.5 mM sodium butyrate treatment. As one of future studies of S-thiolation/dethiolation of rGST P1-1, it deserved to investigate the physiological roles of sulfhydryls of this protein during oxidative stress using rat hepatocyte culture system as a model, and to examine the possible modulation of the activity of this enzyme in vivo by redox reactions such as thiol/disulfide exchange with GSSG. Also in order to have direct information on sites which are responsible for S-thiolation/dethiolation or disulfide bond formation, the crystallization of S-thiolated form of GST P1-1 is necessary.

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GENERAL CONCLUSION

The *in vivo* generation of reactive oxygen species (ROS), may cause detrimental effects on cells because these ROS are known to interact with and alter a wide range of biomolecules including DNA, enzymes and lipids when homeostasis between the rate of ROS generation and ROS disappearance favors the latter, resulting in a state of oxidative stress. These ROS intermediates are suggested to mediate biomolecular interactions which contribute to the initiation, promotion, and/or progression stages of chemical carcinogenesis among other toxic effects.

The cellular free radical scavenger system which is composed of nonenzymatic antioxidants and antioxidant enzymes functions to protect cells from the oxidative damage that occurs. Thus, it is worthwhile to investigate antioxidant food components such as isoflavones as potential anticarcinogens in chemically induced-carcinogenesis, and to study the role of such possible anticarcinogens in altering expression of an antioxidant enzyme defense enzyme that serves as a marker of cancer development. Because of the proposed role of protein S-thiolation /dethiolation as an antioxidant system, the characterization of the thiolation/dethiolation of a protein which is abundant in

neoplasma, and may serve to enhance neoplasm survival as a part of the antioxidant system is also useful.

In this dissertation, rat hepatocarcinogenesis was chosen as a model of carcinogenesis. In chapter 1, fumonisins were used as model cancer promoters, and the expression of two S-thiolatable cytosolic enzymes, the π class of rat glutathione *S*-transferase (rGST P1-1) and carbonic anhydrase III (CA III) were quantified during rat hepatocarcinogenesis. After initiation by diethylnitrosamine (DEN), and during promotion of male rat liver carcinogenesis stimulated by fumonisin treatment, rGST P1-1 level was increased 10 fold, whereas CA III level was suppressed by 43% in hepatic cytosolic fraction. The reasons for an inverse relationship between expression of rGST P1-1 and CA III during cancer development from male rat liver remain to be clarified, but the fact that both rGST P1-1 and CA III have reactive sulfhydryl (-SH) groups, protein -SH groups that are highly reactive with radicals produced during oxidative stress, suggests that rGST P1-1 may replace CA III as an antioxidant protein and that CA III may be a useful biomarker during the development of male rat hepatocarcinogenesis.

In chapter 2, a model system for hepatocarcinogenesis initiated by DEN and promoted by phenobarbital was used, and

soybean isoflavone extract (920 or 1840 μmol/kg diet)fed for 3 and 11 months was examined as a potential component that may be antioxidant anticarcinogenic. After 3 months of PB promotion, both doses of soybean isoflavone extract normalized total hepatic glutathione peroxidase activity (tGPX) which was decreased about 17% by 3 months of PB, and suppressed hepatic tumor promotion as assessed by staining liver for γ-glutamyltransferase (GGT) activity and the presence of rGST P1-1 as biomarkers of altered hepatic foci, suggesting that the anticarcinogenic action of isoflavones is related to the effect of isoflavones upon tGPX. After 11 months of PB promotion, 920 μmol isoflavone extract/kg diet decreased rGST P1-1-[+] AHF compared with the PB-fed group. But neither dose of isoflavone extract suppressed development of GGT-[+] AHF compared with the group fed PB alone. Furthermore, the control group fed 1840 μmol /kg diet of isoflavone extract showed greater development of GGT-[+] and rGST P1-1-[+] AHF than the group fed the basal diet alone. Soybean isoflavones may be antioxidative anticarcinogen, but their margin of safety is relatively narrow, with a cancer-promoting dose of 1840 μmol/kg in female F344/N rats initiated with DEN.

In chapter 3, characterization by isoelectric focusing (IEF) gel/SDS-PA gel electrophoresis and immuno-blot methods of S-thiolation/dethiolation and protein-protein disulfide bond formation of rGST P1-1 was performed using 1) purified rGST P1-1; 2) rat liver extract containing induced rGST P1-1 by a modified Solt-Farber protocol; 3) cultured rat hepatocyte extract containing induced rGST P1-1 by 2.5 mM sodium butyrate treatment. The reaction of purified rGST P1-1 with 20 mM GSSG for 1 or 3 h gave only partially S-thiolated bands, whereas when that of the enzyme with GSSG for 24h, rGST P1-1 showed thiolated bands and no fully reduced band. In addition, 8 and 24 h incubations with 20 mM GSSG revealed a pattern of S-thiolation similar to that obtained after 5 min incubation with 4 or 6 mM diamide, providing further support to the concept that rGST P1-1 contains thiolatable sulfhydryls. Five forms of rGST P1-1 were identified: Band a, fully reduced; band b, mono-glutathiolated; band c, mono-glutathiolated disulfide; band d, di-glutathiolated; band e, di-glutathiolated disulfide form of rGST P1-1. The proposed thiol modifications alter rGST P1-1 catalytic activity because molecular modeling of the three-dimensional structure of GST P1-1 indicated that Cys 47, one of the two most reactive

sulfhydryls is near the active site of the enzyme. Altered rGST P1-1 catalytic activity might therefore occur during oxidative stress associated with cancer development or antineoplastic drug metabolism.

The over expression of rGST P1-1 during rat hepatic carcinogenesis, its selenium independent glutathione peroxidase activity and its ability to undergo S-thiolation/dethiolation and disulfide bond formation suggest that this π class enzyme is a candidate to function as an antioxidant system protecting preneoplastic and neoplastic cells during their growth.

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APPENDIX. THE PURIFICATION OF THE π CLASS OF RAT GLUTATHIONE *S*-TRANSFERASE (rGST P1-1) FROM A MODIFIED SOLT-FARBER TREATED MALE RAT LIVER

The cytosolic homodimeric π class of glutathione *S*-transferases (GST P1-1) whose subunit is 24-24.5 kDa, are known to be early and persistent markers of carcinogenesis. The π class of rat glutathione *S*-transferase (rGST P1-1) was purified by the method of Satoh, K. *et al.* (1985) with some modifications. Rat livers (≥ 20 g) treated with a modified Solt-Farber protocol were homogenized with a Teflon-glass homogenizer in 5 volume of 250 mM sucrose and centrifuged at 10,000 x *g* for 10 min, and the hepatic cytosolic fraction was collected by further centrifugation at 100,000 x *g* at 4°C. The supernatant was dialyzed against 10 mM potassium phosphate buffer containing 2 mM dithiothretol (DTT), pH 6.7 overnight.

The dialyzed sample was applied to a carboxymethyl (CM) cation exchange column (2.8 x 15 cm) that had been equilibrated with the phosphate buffer to absorb basic glutathione *S*-transferases. Unbound or loosely bound neutral and acidic glutathione *S*-transferase were eluted with 3 times the bed volume of the same buffer at a flow rate 0.7

ml/min and then, the flow-through fraction was loaded on *S*-hexyl-glutathione-agarose affinity column (1 x 12 cm) that had been equilibrated with 10 mM Tris, pH 7.8, and washed with 10 mM Tris/0.2 M NaCl, pH 7.8 until no protein was eluted with 5 mM *S*-hexylglutathione dissolved in 10 mM Tris/HCl buffer, pH 7.8 containing 0.2 M NaCl. The active fractions were dialyzed against 10 mM Tris/HCl buffer containing 2 mM dithiothreitol (DTT),pH 8.1 overnight, and subsequently applied to diethylaminoethyl (DEAE)-52 anion exchange column (20 x 1.5 cm) to separate GST P1-1 from the acidic and residual basic forms of glutathione *S*-transferases. Elution was performed at a flow rate of 0.7 ml/min with a NaCl linear gradient from 0 (200 ml) to 100 mM (200 ml). Column effluent was continuously monitored at 280 nm using Shimadzu UV-VIS spectrophotometer UV-140 (Kyoto, Japan), and each fraction was assayed for enzyme activity with 1-chloro-2,4-dinitro-benzene (CDNB). Fractions were analyzed by SDS-polyacrylamide gel electrophoresis to identify which fraction contained GST P1-1. Those fractions containing rGST P1-1 were concentrated by using a Centricon - 3 concentrator (Amicon, Inc. Beverly, MA). This concentrated fraction was incubated in 20 mM β-glycerophosphate, pH 7.5 containing 10 mM DTT for 1 h at

30°C, and dialyzed against 20 mM β-glycerophosphate, pH 7.5 overnight, and was analyzed with isoelectric focusing gel electrophoresis followed by Coomassie blue staining and immnuno-blot with antiserum prepared from the purified protein to check its purity. Finally, this concentrated GST P1-1 was divided into aliquots and stored at -70°C.

Figure 1 shows the elution profile of fractions after the DEAE-52 column. Three major peaks in the elution profile were observed, in which one band having the same migration as the π class of human glutathione *S*-transferase(hGST P1-1) on SDS-polyacrylamide gel electrophoresis was detectable in the pooled and concentrated fractions from the second peak. By this procedure, rGST P1-1 was purified 23 fold (Table 1).

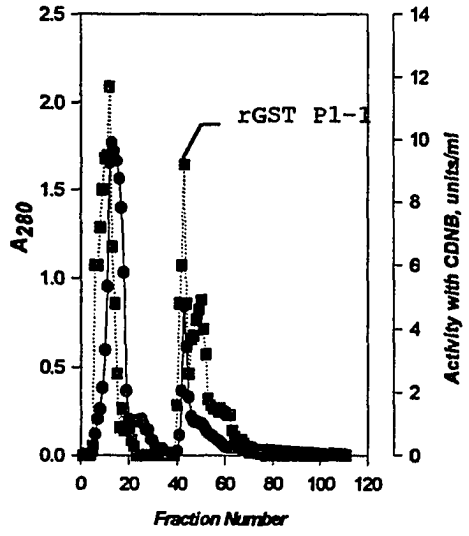

Figure 1. Separation of the π class of rat glutathione *S*-transferase (rGST P1-1) on DEAE-52 cellulose anion exchange column (1.5 x 20 cm)

Rat GST P1-1 was separated from other basic and acidic isoenzymes of GSTs on DEAE-52 cellulose chromatography as described. Two-milliliter fractions were collected and monitored for protein content (A_{280}, -●-)was 2 ml, and the flow rate was 0.7 ml/min. The activity of GSTs was assayed with 1,2-dichloro-4-nitrobenzene (CDNB) (-■-) as a substrate.

Table 1. Purification of rGST P1-1 from modified Solt-Farber treated rat livers.

Step	Vol, ml	Total activity (µmole/min)	Protein mg	Specific Activity (µmole/min per mg protein)	Fold purification
Supernatant at 105,000xg	75	428	965	0.44	-
CM-Sephadex	58	308	580	0.53	1.2
S-hexyl-GSH-agarose	13	200	27.0	6.9	15.7
DEAE-cellulose	12	9.9	0.9	11	23