# Purification, Mass Spectrometric Characterization, and Covalent Modification of Murine Glutathione S-Transferases

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Three cytosolic glutathione S-transferase (GST, EC 2.5.1.18) isozymes were purified from livers of male CD-1 mice to homogeneity using affinity chromatography and gradient elution. Isozyme molecular masses and purities were determined using electrospray ionization mass spectrometry (ESI/MS), HPLC, and electrophoretic methods. Isozymes were assigned to alpha, pi, and mu classes based on kinetic and electrophoretic properties. Molecular masses determined by mass spectrometry were 25 271.4 ± 2, 23 478.8 ± 2, and 25 838.4 ± 2 Da for alpha, pi, and mu isozymes, respectively. Molecular masses for pi and mu class isozymes agreed with molecular masses derived from nucleic acid sequences, but the molecular mass of the alpha class isozyme was 42 Da greater than calculated for a reported sequence. Analysis of tryptic digests of GST-alpha using tandem mass spectrometry determined that the molecular weight discrepancy could be attributed to N-terminal acetylation. Effects of covalent attachment of 4-hydroxy-2-nonenal (HNE) on catalytic activities of each GST isozyme were investigated by incubating GSTs with HNE. The extent of HNE–protein adduct formation was determined using ESI/MS. Catalytic activity of HNE-modified GSTs toward CDNB was measured. ESI mass spectra of modified enzymes show that the extent of HNE modification to the isozymes (pi > alpha > mu) coincides with the loss of activity seen for each protein. ESI mass spectra also indicated that glutathione–protein adducts form in isozymes during incubations with HNE even though purified proteins had been dialyzed against water before incubations. Glutathionylation of isozymes increased as the concentration of HNE in the incubations was raised. Dialysis of GSTs against 2 mM S-hexylglutathione (to displace GSH) before incubations eliminated S-glutathionylation. Reactivities of these GSTs toward HNE differed and is related to the number of cysteine residues (alpha < mu < pi) within the amino acid sequence.

## Introduction

The glutathione S-transferases (GST,[1] EC 2.5.1.18) make up a complex multigene family of proteins that may be the most important in detoxifying electrophilic xenobiotics in nearly all species studied (*1, 2*). The primary function of these proteins is to catalyze the nucleophilic conjugation of reduced glutathione (GSH) to exogenous and endogenous electrophiles (for reviews, see refs *3–5*). GSTs play key roles in metabolism of chemotherapeutic agents and prevention of chemical carcinogenesis (*6*), resistance of tumors to therapeutic drugs (*7*), resistance of insects to selected pesticides (*8*), and selective action of herbicides (*9*). Species- and tissue-selective expression of the various forms of GSTs is influenced by genetic, hormonal, and environmental factors (*10–12*). Understanding the expression, structures, and functions of individual GST isozymes in various tissues is critical to determining mechanisms of species- and tissue-selective toxicity.

GSTs exist in numerous forms, each of which exhibits a broad but characteristic substrate specificity. GST isozymes exist as catalytically active dimers with subunits of about 25 kDa. GSTs have been categorized into three principal classes (alpha, mu, pi) based on physical, immunological, and kinetic properties and sequences (*13*). In the mouse, both cytosolic and microsomal forms of GSTs have been characterized (*14–19*) and exhibit strain-dependent differences in their sequences (*18*). Gender-related differences in the expression and kinetic properties of mouse GST isozymes have also been observed (*20*).

Due to immunochemical cross-reactivity within classes of GSTs (*21, 22*), IEF and SDS–PAGE electrophoretic methods find routine use for distinguishing isozymes. However, molecular masses and p*I*s reported for similar isozymes exhibit substantial variability, and misclassification of GST isozymes can result from variability in the composition of the resolving gel (*23*). Oxidation, degradation, and covalent modification of isozymes can also occur during purification, generating additional bands that can be erroneously interpreted to be new isozymes (*10*). Reverse-phase HPLC methods have been described for the separation and quantitation of GST subunits (*24*).

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[1] Abbreviations: GST, glutathione S-transferase; IEF, isoelectric focusing; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ESI, electrospray ionization; MS/MS, tandem mass spectrometry; HNE, *trans*-4-hydroxy-2-nonenal; CDNB, 1-chloro-2,4-dinitrobenzene; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; TFA, trifluoroacetic acid.

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Most information about GST sequences has come from cDNA sequences, but errors in nucleic acid sequencing are common (*25*). Furthermore, posttranslational processing of proteins is not evident from cDNA sequences, and such considerations become important when GSTs are expressed in recombinant systems. Differences in physical properties such as p*I* values have been reported in comparing native GSTs with recombinant GSTs (*19*).

Recent advances in ionization techniques for mass spectrometry provide the capability to resolve ambiguities left by traditional sequencing methods. The development of electrospray ionization mass spectrometry (ESI/MS) has made possible the accurate determination of protein molecular masses and the elucidation of posttranslational modifications including covalent modifications by reactive electrophiles (for reviews, see refs *26* and *27*). The ESI process results in the evaporation of ionized protein molecules with a distribution in the numbers of attached protons and, thereby, a distribution in charge states. A mass spectrometer separates the ionized molecules according to their mass-to-charge ratios and determines amounts of each ion produced. Molecular masses of proteins of 30 kDa have been measured to within ±0.01% of theory, which is 3 orders of magnitude superior to the ±10% typical of SDS–PAGE (*27*). ESI/MS methods also exhibit resolution capable of distinguishing differences in molecular masses of less than 1% in protein mixtures allowing for identification of variant proteins (*28, 29*) and posttranslational modifications (*30, 31*). Sites of protein modification have been determined using tandem mass spectrometry (*32*).

A promising application of mass spectrometry focuses on characterization of protein–electrophile conjugates. An electrophile that has attracted great interest is the cytotoxic lipid peroxidation product *trans*-4-hydroxy-2-nonenal (HNE). This reactive aldehyde inactivates a variety of enzymes including glyceraldehyde-3-phosphate dehydrogenase (*33*) and alcohol dehydrogenase (*34*), and several α,β-unsaturated carbonyl compounds inhibit cytosolic GSTs (*35*). In contrast, several covalent modifications of microsomal GSTs increase the catalytic rate (*36–38*).

The objectives of this present study have been to develop an affinity chromatography method for the isolation and purification of the major GST isozymes present in mouse liver, to characterize the purified isozymes by accurate determination of molecular masses using ESI/MS, and to use this information to verify GST amino acid sequences determined from nucleic acid sequences and use tandem mass spectrometry to identify posttranslational modifications. Secondary goals have been to use this same methodology to characterize covalent modifications to isolated GST isozymes by HNE and explore the relationship between covalent modification of GST isozymes and catalytic activity using the substrate 1-chloro-2,4-dinitrobenzene (CDNB).

## Experimental Procedures

**Caution:** *The range of toxicological effects of HNE are not fully established. This cytotoxicity and mutagenicity of this compound have been noted in earlier studies. Precautions should be taken to minimize exposure.*

**Chemicals.** Ethylenediaminetetraacetic acid (EDTA) disodium salt, sodium chloride, sodium phosphate monobasic, tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), dithiothreitol (DTT), urea, and acetonitrile (Optima grade) were purchased from Fisher Scientific Corp. (Fair Lawn, NJ). Reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene, GSH–agarose (attached through the sulfur to epoxide-activated 4% cross-linked beaded agarose), iodoacetamide, pharmalytes (pH 3–10), L-1-(tosylamino)-2-phenylethyl chloromethyl ketone-treated trypsin, and all protein standards were bought from Sigma Chemical Co. (St. Louis, MO). Dye reagent for protein assays was purchased from Bio-Rad (Hercules, CA). Ionate grade trifluoroacetic acid (TFA) was from Pierce (Rockford, IL). The *S*-hexylglutathione was synthesized from GSH and 1-iodohexane according to the method of Mannervik and Guthenberg (*39*). The HNE was graciously supplied by Bernd Bruenner, Facility for Advanced Instrumentation, University of California–Davis. All other chemicals used were of analytical grade.

**Animals.** Male CD-1 mice, 25–30 g, were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA). Animals were housed on autoclaved pine shavings in approved animal care facilities and fed *ad libitum* for 1 week prior to cervical dislocation.

**Enzyme Assays.** GST activity was measured using GSH and CDNB as substrates according to the method of Habig et al. (*40*). The activity of the enzyme was determined in a 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM GSH and 1 mM CDNB using an extinction coefficient of 9.6 $mM^{-1}$ $cm^{-1}$. One unit of enzyme is defined as the amount required to catalyze the conjugation of 1 μmol of substrate to GSH per minute at 25 °C. The rate of product formation was monitored by measuring the change in absorbance at 340 nm using a Shimadzu PC-2101 UV–visible spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). Specific activities are based on protein concentrations as determined by the method of Bradford (*41*) using bovine serum albumin as a reference.

**Buffers.** Buffers used for protein purification were prepared at 20 °C, and pH values were measured at 4 °C. A variety of buffer solutions were required for purification of the GST isozymes. Buffer compositions were as follows: (A) 10 mM Tris-HCl (pH 7.4); (B) 10 mM Tris-HCl (pH 7.4) containing 250 mM sucrose and 1 mM each of DTT and EDTA; (C) 25 mM phosphate buffer (pH 7.0) containing 1 mM each of DTT and EDTA; (D) 25 mM phosphate buffer (pH 7.0) containing 500 mM NaCl and 1 mM each of DTT and EDTA; (E) 25 mM phosphate buffer (pH 7.4) containing 75 mM GSH, 500 mM NaCl, and 1 mM each of DTT and EDTA; (F) 25 mM phosphate buffer (pH 9.5) containing 75 mM GSH, 500 mM NaCl, and 1 mM each of DTT and EDTA.

**Purification of GST Isozymes.** Mice were killed by cervical dislocation, and livers were removed, rinsed with ice-cold buffer A, and combined. The tissues were homogenized using a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY) set at 70% maximum speed for 1 min on ice (1 g of tissue/2 mL of buffer) in buffer B. Centrifugation and all subsequent purification steps were carried out at 4 °C. The homogenate was centrifuged at 10000*g* for 30 min to pellet cellular debris. The supernatant fraction was collected and centrifuged at 100000*g* for 80 min to remove microsomal GST, leaving cytosolic forms of GSTs in the supernatant. Supernatant was next passed through a Sephadex G-25 column (100 cm × 2.5 cm) which had been pre-equilibrated in buffer C. Column eluants were monitored at 280 nm using an Isco-UA5 detector. Three peaks were eluted from the G-25 column using buffer C. The first of these peaks was the only one found to contain activity toward CDNB and was applied onto a GSH–agarose affinity column (11 cm x 2.5 cm) equilibrated in buffer C. After sample was loaded on the affinity column, the column was washed with buffer C until the baseline was reestablished. The column was next washed with 500 mL of buffer D. This wash removes a large peak of nonspecifically bound proteins which exhibited no detectable activity toward CDNB.

The first two GST isozymes were eluted using a linear gradient from buffer D to buffer E (250 mL × 250 mL). The composition of the elution buffer was then held constant with the addition of an additional 200 mL of buffer E. The addition of this buffer allowed for the complete separation of the second peak from the next eluting peak. The third peak containing

**Table 1. Activities of Glutathione *S*-Transferases toward 1-Chloro-2,4-dinitrobenzene at Various Stages of Isozyme Isolation**

| purification step | total protein[a] (mg) | total units[b] | specific activity[b] (units/mg) | % of original activity | purification factor[d] |
|---|---|---|---|---|---|
| (1) 100000*g* spin | 1654 | 6339.4 | 3.8 | 100 | 1 |
| (2) Sephadex G-25 | 1523 | 5009.2 | 3.3 | 79 | 0.86 |
| (3) GSH-linked agarose | | | | | |
| non-retained material | nd[e] | 198 | | 3 | |
| P1 | 9.8 | 630 | 64 | 10 | |
| P2 | 5.8 | 859 | 148 | 14 | |
| P3 | 14.8 | 1098 | 74 | 17.3 | |
| P4 | 14.1 | 3440 | 244 | 54.3 | |
| total recoveries[c] | 44.5 | 6225 | | 98.6 | |

[a] Protein determined according to the method of Bradford using bovine serum albumin as reference. [b] One unit of enzyme activity is defined as the amount of enzyme which catalyzes the formation of 1 $\mu$mol min$^{-1}$ of *S*-(2,4-dinitrophenyl)glutathione under routine assay conditions at 25 °C. [c] Total recoveries based upon combined pools P1, P2, P3, and P4 plus material not retained by the affinity column. [d] Purification factors calculated as the ratio of the pool specific activity to the specific activity of the initial supernatant after centrifugation. [e] nd, not determined.

activity toward CDNB was eluted using a linear gradient from buffer E to buffer F (150 mL × 150 mL).

The three main peaks of activity were recovered and pooled separately. Each pool was concentrated to 25–30 mL in an Amicon ultrafiltration cell (Amicon, Inc., Beverly, MA) with a PM 10 membrane and dialyzed against 4 L of 1 mM DTT in nanopure water overnight followed by dialysis against 3 changes (4 L each) of nanopure water. Enzymatic activity toward CDNB was measured on freshly dialyzed pools. Aliquots were removed from pools for analysis using IEF, and dialyzed concentrates were lyophilized and stored at −80 °C.

**Electrophoretic Analysis.** SDS–PAGE was carried out according to the method of Laemmli (*42*), using a resolving gel comprised of 10% polyacrylamide and 2.7% bis(acrylamide) and a stacking gel comprised of 4% polyacrylamide and 2.7% bis(acrylamide). Proteins of known subunit molecular weight served as standards (lysozyme 14 300, trypsin inhibitor 20 100, carbonic anhydrase 29 000, alcohol dehydrogenase 39 800, bovine serum albumin 66 000, phosphorylase *b* 97 400). Isoelectric-point determination was carried out using a Pharmacia Multiphore II system and gels comprised of 6% polyacrylamide and 2.7% bis(acrylamide) containing 2% pharmalytes of pH range 3–10. Protein standards used were as follows: trypsin inhibitor, $\beta$-lactoglobulin, carbonic anhydrase, myoglobin, lactate dehydrogenase, trypsinogen, and cytochrome *c*.

**HPLC Analysis of GSTs.** The subunit compositions of affinity purified GSTs were characterized using reverse-phase HPLC on a Vydac 218TP54 (25 cm x 4.6 mm i.d., 300 Å pore size) C-18 column. Chromatographic analyses were carried out using a Varian 9010 solvent delivery system and a Varian 9050 variable wavelength UV–visible detector monitoring absorbance at 214 nm. Proteins were separated at a flow rate of 1.0 mL/min with an analysis time of 30 min/sample. The initial mobile phase composition was held at 40% acetonitrile/60% $H_2O$ containing 0.1% TFA (v/v) for 10 min followed by a 20 min gradient to 60% acetonitrile/40% $H_2O$ containing 0.1% TFA (v/v).

**Mass Spectrometry.** Lyophilized proteins were dissolved in 50/50 ACN/water to give a final concentration of 1 $\mu$g/$\mu$L (40 pmol/$\mu$L). Intact proteins were analyzed on a VG/Fisons Quattro-BQ triple quadrupole mass spectrometer (VG Biotech, Altrincham, U.K.) using 50/50 ACN/water + 1% formic acid as the mobile phase. An Isco $\mu$LC-500 syringe pump delivered the mobile phase at 10 $\mu$L/min. Proteins were analyzed by direct flow injection using an injection volume of 10 $\mu$L. Spectra were obtained in positive ion mode using a capillary voltage of +3.5 kV, and the source temperature was held at 65 °C. The cone voltage was set between 35 and 50 V. Spectra were scanned over the range of $m/z$ 600–1400 at 10 s/scan and summed using the MCA acquisition mode in the Fisons Masslynx software. Mass calibration was performed using horse heart myoglobin as reference. ESI MS/MS experiments were conducted on tryptic digests of GSTs using argon as the collision gas at a measured pressure of 1 × $10^{-3}$ mbar, and the collision cell was floated at −20 V.

**Tryptic Digestion.** Procedures for tryptic digestion of GST isozymes followed published methods (*43*). ESI/MS analysis is best performed when salts are removed from samples. Therefore, salts remaining in samples after digestion were removed by loading digest fragments onto a $C_{18}$ Bond Elut solid phase extraction column (1 cm$^3$, Varian, Sunnyvale, CA). The column was rinsed several times with nanopure water containing 0.1% TFA (v/v) to remove salts. Digest fragments were eluted in 3 column volumes of acetonitrile in 0.1% TFA (v/v). Samples were then concentrated to ~200 $\mu$L under a stream of nitrogen at room temperature. Aliquots were removed and mixed with an equal volume of water for ESI MS/MS experiments.

**GST Modification.** Affinity purified isozymes were dialyzed against 4 changes of nanopure water and lyophilized. Proteins were reconstituted at a concentration of 40 $\mu$M (about 1 mg/mL) in 0.1 M sodium phosphate buffer (pH 7.4) and incubated for 3 h at 37 °C in a shaking water bath with 1-, 2-, 5-, and 10-fold molar equivalents of HNE. Proteins were then dialyzed against 3 changes (4 L) of nanopure water and lyophilized. Protein content and activity toward CDNB was measured after dialysis so that changes in the specific activity of the isozymes that could be attributed to modification could be determined. Proteins were lyophilized, frozen at −80 °C and archived for characterization using ESI/MS.

## Results and Discussion

**Enzyme Purification.** A summary of the yields and activities of the GSTs obtained from mouse liver cytosol is presented in Table 1. Purification of GST on the GSH-linked agarose resulted in a purification of isozymes to greater than 91% homogeneity according to both ESI/MS and HPLC data with minimal loss of total enzyme activity. Three percent of the total enzyme activity applied to the affinity column passed through unretained. Gradient elution of GST isozymes from the affinity resin yielded three predominant peaks that exhibited activity toward CDNB (Figure 1). Fractions corresponding to these peaks were pooled and designated P1, P3, and P4 in the order in which they eluted from the affinity resin. An elevated baseline was observed to occur between P1 and P3. To establish if this elevation was due to a trailing of P1 or a separate isozyme, fractions corresponding to this region were pooled separately and designated as P2. The splitting of the peak corresponding to P3 of the activity profile resulted from the addition of new buffer minutes after first gradient was complete.

This simplified two-step affinity purification resulted in a 98% recovery of the total GST activity applied to the affinity column. The sequential use of gradients in salt concentration, pH, and GSH concentration results

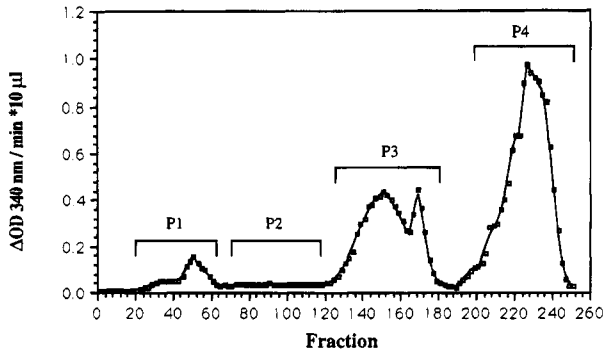


**Figure 1.** Preparative affinity chromatogram of cytosolic glutathione *S*-transferases isolated from mouse liver. Activity was determined using CDNB as substrate. Pool 1 consisted of fractions 20–60, pool 2 of fractions 66–116, pool 3 of fractions 124–180, and pool 4 of fractions 192–250.

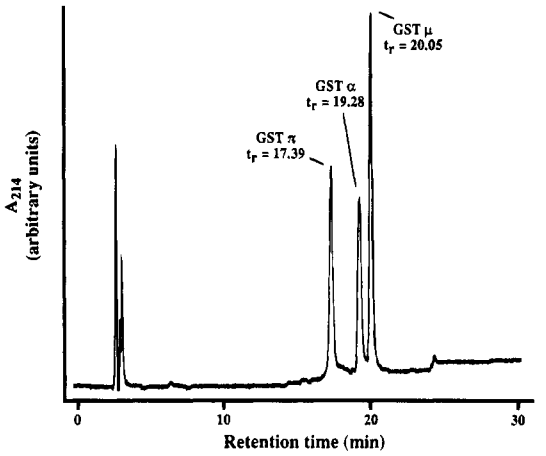


**Figure 2.** Reverse-phase HPLC chromatographic separation of murine glutathione *S*-transferase subunits for combined pools of isolated GST isozymes.

in the complete separation of the three predominant cytosolic classes of GST isozymes of mouse liver with high specific activities. This purification method is a relatively fast (it can be completed in 24 h) and simple procedure (low pressure column chromatography) for the preparative isolation of mouse liver GST isozymes in comparison to earlier methods developed (*15–17*).

**Enzyme Characterization.** Fractions collected during the affinity purification were profiled using CDNB (Figure 1) and substrates specific to each isozyme (data not shown). The results of these studies indicate that pools P1 and P2 exhibit nearly all of the non-selenium-dependent peroxidase activity as shown by their activity toward cumene hydroperoxide. Transferase P3 demonstrated the highest specific activity toward ethacrynic acid, and the pooled fractions corresponding to P4 showed the highest specific activity toward bromosulfophthalein. On the basis of these results, we tentatively identified P1 and P2 as containing alpha class isozymes and P3 and P4 as pi and mu class isozymes, respectively.

The apparent molecular mass values and isozyme purities of the pooled isozymes were estimated using SDS–PAGE. The results of this experiment indicate that P1, P3, and P4 consist of only one subunit each with apparent molecular mass values of 26 000, 24 500, and 27 000 Da, respectively. When these purified pools were subjected to IEF, each yielded a single band with p*I*s of 9.2 (P1), 8.7 (P3), and 8.3 (P4). P2 was not distinguishable from P1 using either SDS–PAGE or IEF.

Reverse-phase HPLC of each the pooled fractions from the affinity purification resulted in a single major peak, confirming that each isozyme was a homodimer. Pools P1 and P2 were 91% and 93% pure, respectively, with the major component eluting with a retention time of 19.30 min. Pool P3 was 98% pure with the major component eluting at 17.37 min, and pool P4 was 93% pure with the most abundant component eluting at 20.24 min. The minor contaminating peaks which correspond to less than 10% of each parent peak were also seen in the ESI/MS spectra for P1 and P4. These spectra showed a difference in molecular mass of the minor peak of 357 Da for the alpha class GST and 353 Da for the mu class GST. The molecular masses of these minor peaks do not correspond to the molecular masses of other known GST isozymes and therefore are more likely the result of a modification to the parent protein which occurred during the purification process (e.g., glutathionylation). A chromatogram of the combined pools is shown in Figure 2.

To further characterize the purified GST isozymes, our results were compared with those obtained in several laboratories that have isolated similar cytosolic GST isozymes purified from mouse livers (*15–17*, *44*, *45*), and the results are summarized in Table 2. These earlier studies isolated and characterized liver cytosolic GSTs from various strains of mice including both males and females. The results from the current study show best agreement with the molecular masses and p*I*s determined by Hayes et al. (*44*), but inter laboratory uncertainties in SDS–PAGE and IEF analyses are too great to resolve the small differences between reported GST sequences, particularly within specific isozyme classes.

**Electrospray Characterization of GST Isozymes.** Pooled GST isozymes from the affinity purification were characterized by ESI/MS. The ESI/MS spectra characterization of isolated GST isozymes was found to be a fast and straightforward method for determining isozyme molecular masses and indicating purity. The ESI mass spectra are reproducible, and precise measurement of molecular masses (standard deviation = ±2 Da; $n = 3$) is easily obtained for purified GSTs. ESI/MS is able to yield molecular masses of GST proteins to within 0.008% of theory. The following molecular masses for each pool are reported as averages of 3 separate determinations. The molecular mass of P1 and P2 was determined as 25 271.4 ± 2 Da. A minor peak (intensity less than 10% of the base peak) was also seen at 25 629.5 Da. The respective molecular mass of P3 and P4 were determined as 23 478.8 ± 2 and 25 838.4 ± 2 Da. P4 contained a minor peak (again intensity less than 10% of the base peak) which corresponded to a molecular mass of 26 191.2 Da. ESI/MS spectra for P1, P3, and P4 can be found in Figures 3a, 4a, and 5a. Mathematical transformation of these data using the Maximum Entropy algorithm (Fisons Masslynx software) provides spectra which represent the true mass scale and are shown in Figures 3b, 4b, and 5b, respectively.

By comparing the molecular masses obtained using ESI/MS to molecular weights calculated from published nucleic acid sequences, it was possible to determine whether published sequences agreed with experimental molecular masses without ambiguity. The molecular mass of P3 is in agreement with the molecular mass derived from a nucleic acid sequence for the pi class isozyme (calcd 23 478.0 Da) from BALB/C strain of mice as reported by Hatayama and coworkers (*46*). The molecular mass of P4 also agrees with a nucleic acid

**Table 2. A Comparison of Molecular Weights and Isoelectric Points for Glutathione *S*-Transferases Isolated from Mouse Liver Cytosol Determined in This Study and from Earlier Studies**

| | | alpha | | pi | | mu | |
|---|---|---|---|---|---|---|---|
| ref | strain | molecular mass (Da) | p*I*[c] | molecular mass (Da) | p*I*[c] | molecular mass (Da) | p*I*[c] |
| this study | CD-1 (male) | 26 000[a]<br>25271 ± 2[b] | 9.2 | 24 500[a]<br>23479 ± 2[b] | 8.7 | 27 000[a]<br>25838 ± 2[b] | 8.3 |
| Lee, et al. (*15*) | DBJ/2J | 22 000[a] | 6.5 | 22 000[a] | 8.2 | 25 000[a] | 8.8 |
| Warholm et al. (*17*) | NMRI | 25 000[a] (MI) | 9.7 | 23 000[a] (MII) | 8.7 | 26 000[a] (MIII) | 8.5 |
| Hayes et al. (*44*) | n/rd | 26 000[a] (Ya) | 9.2 | 24 500[a] (Yf) | 8.6 | 27 000[a] (Yb) | 7.8–8.2 |
| McLellan and Hayes (*18*) | DBA/2, C3H/He, C57BL6 (both genders) | 25 800[a] (Ya) | ≥9.2 | 24 800[a] (Yf) | 8.7 | 26 400[a] (Yb) | 7.8–8.2 |
| McLellan and Hayes (*45*) | BALB/c, 129/OLA (both genders) | 25 600[a] (Ya1)<br>25 800[a] (Ya3) | n/r | 24 800[a] (Yf) | n/r | 26 400[a] (Yb) | n/r |
| Pearson et al. (*16*) | CD-1 (female) | n/r | n/a | n/r | n/a | 24 000[a]<br>24 000[a] | 8.7<br>9.3 |

[a] Determined for GST monomers using SDS–PAGE (uncertainties not reported). [b] Determined for GST monomers using electrospray ionization mass spectrometry. [c] p*I* values presumed to be for homodimeric forms of GSTs. [d] n/r, not reported; n/a, not applicable.

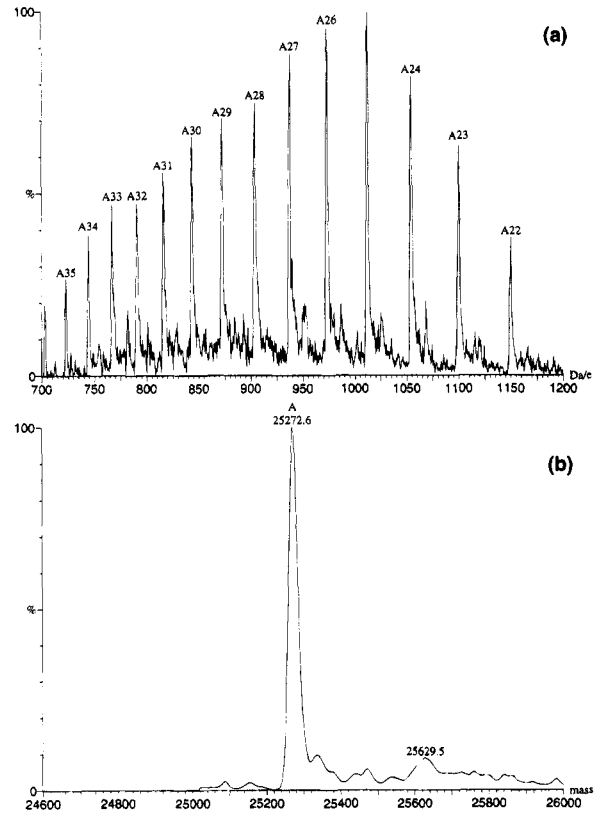


**Figure 3.** Positive mode electrospray ionization mass spectrum (a) and true mass scale Maximum Entropy transformed spectrum (b) of alpha class glutathione *S*-transferase from pool 1 (P1).

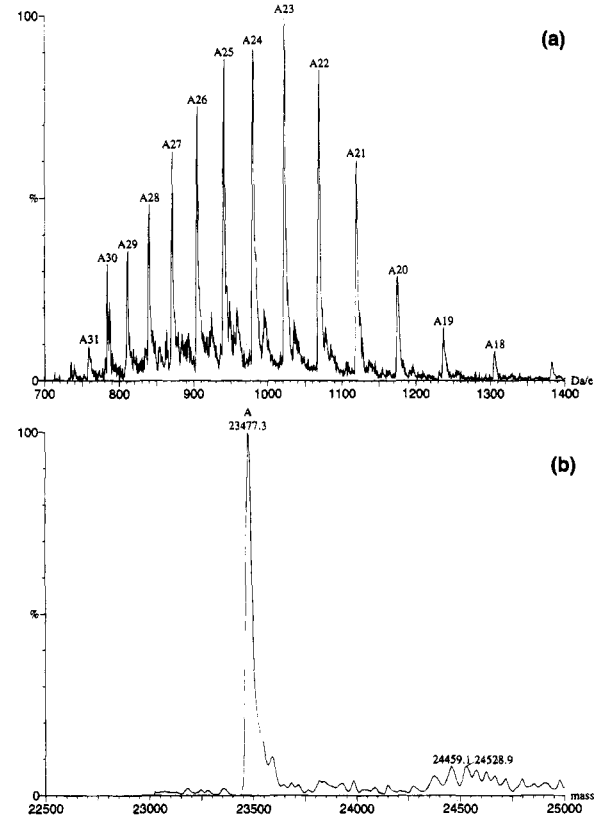


**Figure 4.** Positive mode electrospray ionization mass spectrum (a) and true mass scale Maximum Entropy transformed spectrum (b) of pi class glutathione *S*-transferase from pool 3 (P3).

sequence reported by Pearson and co-workers (*47*) for a mu class (calcd 25 838.8 Da) isozyme, but the molecular mass determination for the P1 (and P2) isozyme did not match the molecular masses derived from any nucleic acid sequences published to date. However, the molecular mass determined by ESI/MS is 42 Da greater than the value calculated (calcd 25 229.0 Da) for a sequence of an alpha isozyme isolated from BALB/C mice by Hayes et al. (*48*). The difference of 42 Da can be explained as arising from posttranslational N-acetylation of the N-terminal amino acid. An earlier study of GSTs isolated from outbred NMRI strain mice reported that the N-terminus of the alpha isozyme (referred to as GST MI) was indeed blocked (*13*).

**ESI MS/MS Characterization of GST Peptides.** One of the most compelling applications of ESI/MS is in the identification of posttranslational modifications in proteins. Tandem mass spectrometry (MS/MS) involves inducing ionized molecules to fragment, usually via collision with a target gas such as argon, and analyzing the fragments with a second mass analyzer. In the case of peptides, much of the fragmentation occurs at the peptide bonds, and fragment masses can be used to establish amino acid sequences and the location of posttranslational modifications.

The ESI mass spectrum of products of tryptic digestion of the alpha class GST (P1) is shown in Figure 6A. Because tryptic fragments usually contain a basic amino acid residue at the C-terminus, the formation of doubly-charged $[M + 2H]^{2+}$ ions upon electrospray ionization is common. Using the reported sequence (*48*), we were able to identify a prominent doubly-protonated digest fragment appearing at $m/z$ 701.5. This doubly-protonated

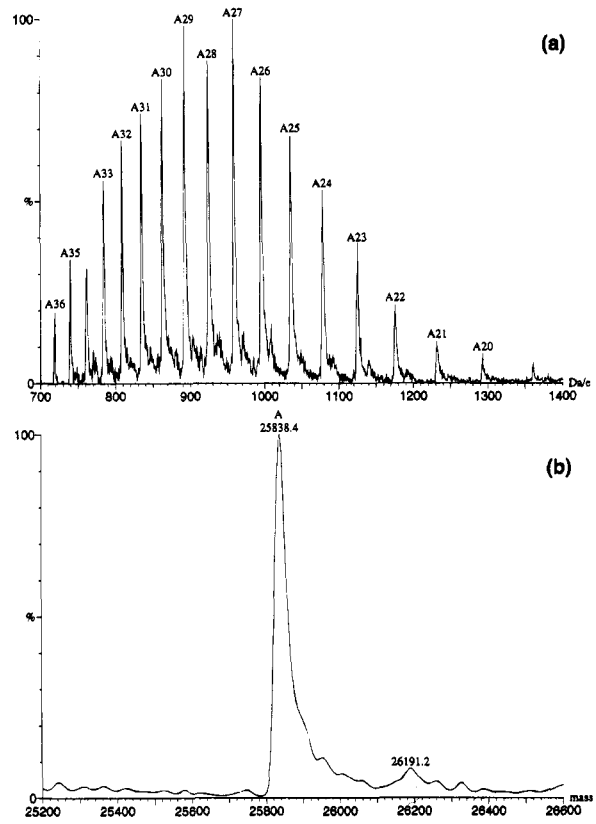


**Figure 5.** Positive mode electrospray ionization mass spectrum (a) and true mass scale Maximum Entropy transformed spectrum (b) of mu class glutathione *S*-transferase from pool 4 (P4).

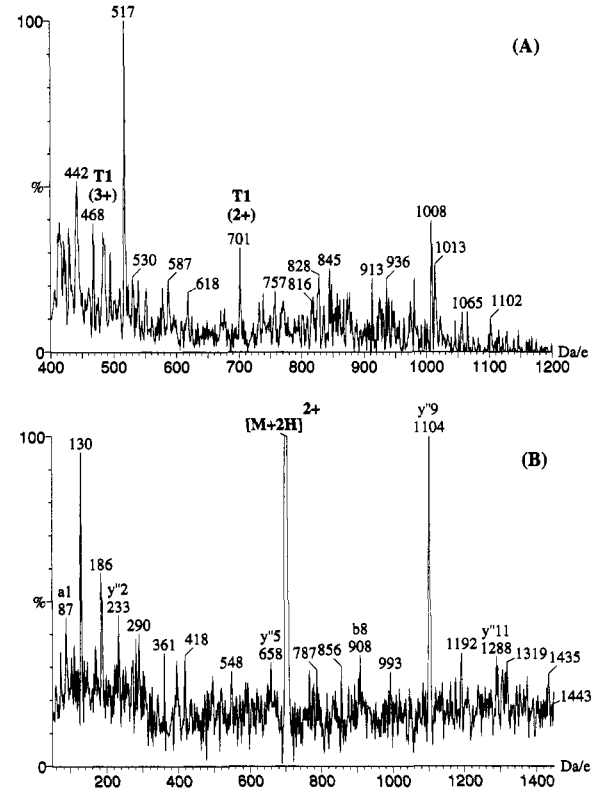


**Figure 6.** (A) Positive mode electrospray ionization mass spectrum of products of tryptic digestion of purified alpha class GST (P1) and (B) daughter spectrum of $m/z$ 701.5 (the doubly-protonated tryptic fragment T1).

fragment corresponds to an N-acetylated sequence of the N-terminal tryptic fragment T1 (e.g., *N*-acetyl-AGKPV-LHYPEGR) which has a molecular mass of 1401.6 Da. A peak corresponding to the triply-protonated T1 fragment can also be observed at $m/z$ 468.

MS/MS experiments were performed on the mixtures of tryptic digestion products by isolating ions of $m/z$ 701.5 and inducing them to fragment via collisional activation. The spectrum of fragments (product ion spectrum) for this doubly-charged peptide is shown in Figure 6B. This spectrum has two key features that indicate N-terminal acetylation. The first is the presence of the product ion at $m/z$ 1104, which is a singly-charged fragment corresponding to loss of the residues Ac-AGKP. Fragmentation would be expected to be facile here due to the strained conformation of the peptide at the linkage to proline. Further evidence of the acetylated N-terminus is found in the form of a product ion peak at $m/z$ 130, which is indicative of a alanine residue that has been N-acetylated. Numerous additional product ions provide additional support for the proposed sequence.

**Glutathione *S*-Transferase Modification.** CDNB activities of the lyophilized GSTs incubated with 1-, 2-, 5-, and 10-fold molar equivalents of HNE can be found in Figure 7. Decreases in the activity of the alpha class GST isozyme occurred with only 5- and 10-fold molar equivalents of HNE, and no change in activity was discernible after incubation with 1 or 2 molar equivalents of HNE. ESI mass spectra for the incubation of the alpha class GST with 1 and 2 molar equivalents of HNE indicated that only minor amounts (less than 2%) of the protein were modified. The pi class isozyme exhibited greater reactivity toward HNE, and its activity decreased in inverse proportion with the increase in HNE concentration. A near complete loss in activity was observed upon incubation with 10 molar equivalents of HNE. ESI mass spectra indicated that less than 11% of the protein remained in its native form at this concentration of HNE. The mu class isozyme showed only a minimal decrease in activity as measured with CDNB at even the highest molar ratio of HNE/protein. ESI mass spectra for this isozyme indicated that very little covalent modification of the protein had occurred.

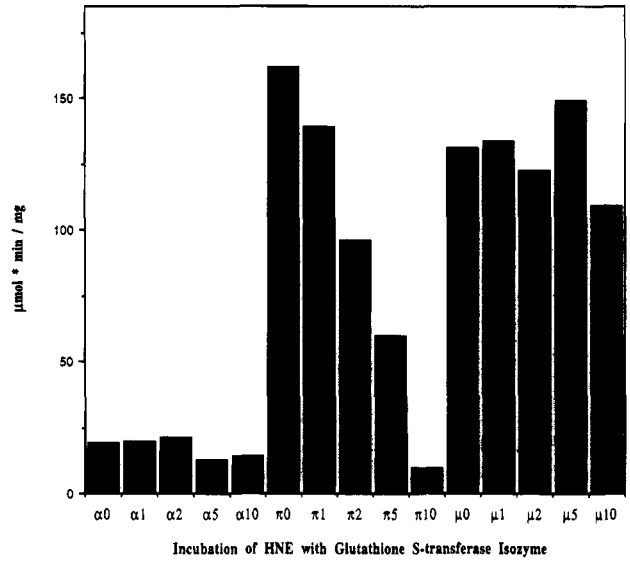


**Figure 7.** Effects of incubation of GST isozymes with various amounts of 4-hydroxy-2-nonenal upon catalytic activity toward CDNB. Incubation conditions are noted along the horizontal axis, indicating the isozyme and the molar ratio of HNE:protein.

ESI/MS spectra of GST isozymes alpha, pi and mu incubated with a 5-fold molar excess of HNE are shown

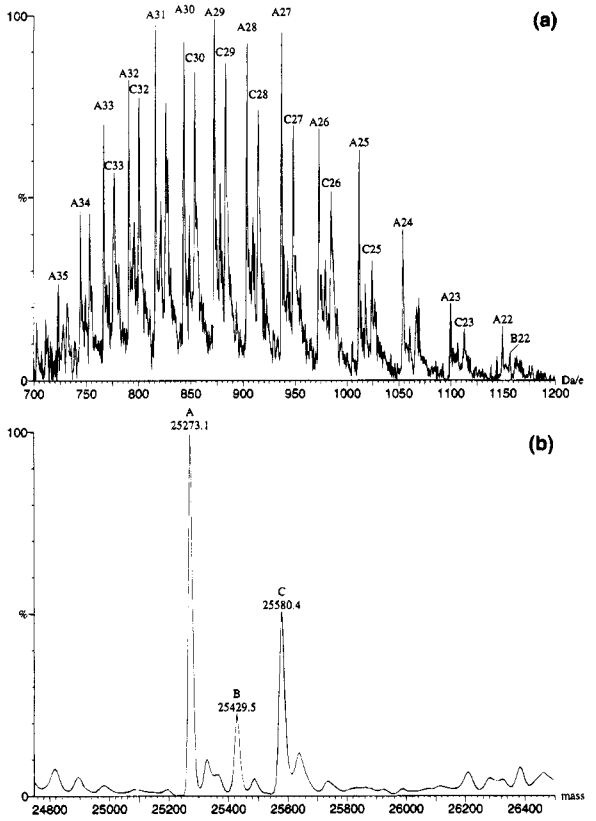


**Figure 8.** Positive mode electrospray ionization mass spectrum (a) and true mass scale Maximum Entropy transformed spectrum (b) of alpha class glutathione *S*-transferase modified with a 5-fold molar equivalent of HNE.

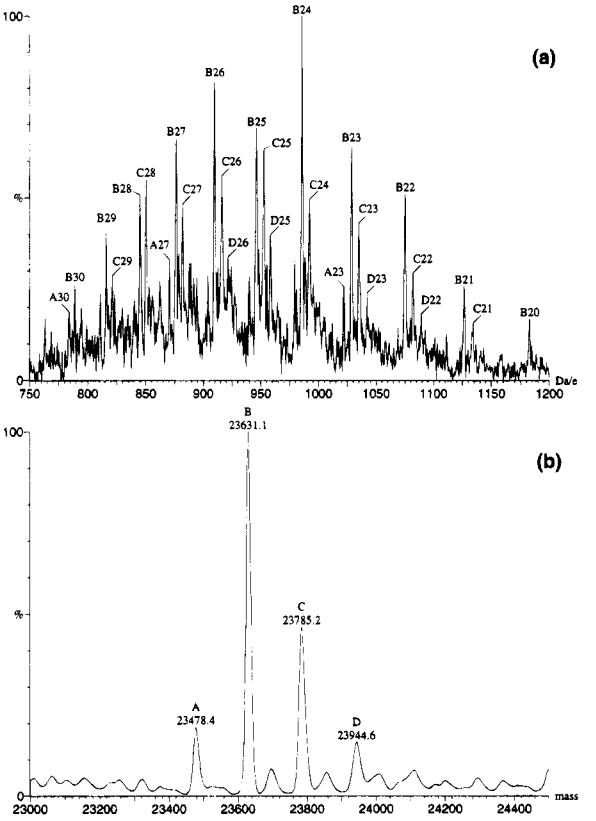


**Figure 9.** Positive mode electrospray ionization mass spectrum (a) and true mass scale Maximum Entropy transformed spectrum (b) of pi class glutathione *S*-transferase modified with a 5-fold molar equivalent of HNE.

in Figures 8a, 9a, and 10a, respectively. Maximum Entropy transformed (true mass scale) ESI/MS spectra for these isozymes are shown in Figures 8b, 9b, and 10b. ESI mass spectra indicate that both HNE–protein and glutathione–protein adducts form in all three isozymes during incubations with HNE even though purified proteins had been dialyzed against nanopure water before incubations. The presence of each HNE group causes an increase of 156 Da in molecular mass, occurs almost exclusively via Michael addition, and is not readily reversible (*33, 49*). The presence of a glutathione disulfide adduct results in an increase of 305 Da in molecular mass. In all three isozymes the extent of both glutathione– and HNE–protein adducts increased as the concentration of HNE in the incubation was raised.

ESI mass spectra of the alpha class GST incubated with a 5-fold molar excess of HNE indicate that both HNE and glutathione adducts are formed (Figure 8). Peak A (Figure 8b) corresponds to the native protein while peak B corresponds to the addition of one HNE and peak C agrees with the formation of one glutathione disulfide adduct. The intensity of peak C indicates that glutathionylation of the alpha class GST occurred to a greater extent than HNE–protein adducts. Both modifications increased as the HNE:protein ratio was raised in the incubations. In an earlier study, affinity purified isozymes which were dialyzed overnight against 2.0 mM *S*-hexylglutathione (to displace residual bound GSH) before incubation with HNE showed no evidence of glutathionylation. In this study, ESI mass spectra indicated that the extent of HNE modification for the different isozymes paralleled the number of cysteine residues present within their amino acid sequences (alpha < mu < pi).

Efforts to characterize the sites of modification were made by performing tryptic digestion of GST-alpha that had been incubated with a 10 molar equivalents of HNE. Tryptic digestion of modified GST proceeded slowly and not to completion. HPLC fractions of tryptic digests were analyzed by ESI mass spectrometry, and the mass spectra showed the presence of some unmodified fragments, but the fragment containing Cys was not seen either with or without attached HNE due to incomplete digestion. Determination of sites of GST modification is the subject of continuing investigations.

The presence of glutathionylated and HNE-modified GST was also evident from spectra obtained for the pi and mu class isozymes. The pi class isozyme was extensively modified by HNE and to a lesser extent by glutathione, with a 5-fold molar excess of HNE, as can be seen in peaks B and C of the spectrum in Figure 9b. Peak D of this spectrum corresponds to the addition of three HNE moieties. Again, the extent of HNE modification of the pi class GST (~89% of the total was modified) corresponds with the near complete loss in activity toward CDNB. The mu class GST was not extensively modified by HNE or glutathione at a 5-fold molar excess of HNE as can be seen in peaks B and C of Figure 10b. The limited amount of modification to the mu class isozyme coincides well with the maintenance of CDNB activity as seen with this isozyme.

It is important to note that the inactivation of GSTs by HNE does not appear to involve intramolecular crosslinking through the aldehyde functional group. Such condensations involve loss of water and a decrease in molecular mass of 18 Da, but no evidence of this decrease in molecular mass was observed in the ESI mass spectra. Similar inactivation of pi class GSTs by *N*-ethylmaleim-

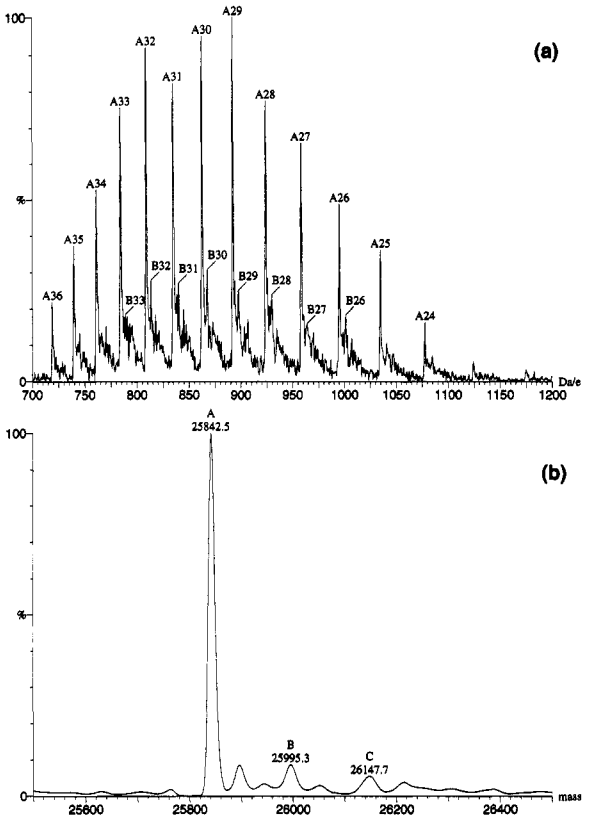


**Figure 10.** Positive mode electrospray ionization mass spectrum (a) and true mass scale Maximum Entropy transformed spectrum (b) of mu class glutathione *S*-transferase modified with a 5-fold molar equivalent of HNE.

ide was observed in an earlier study by Tamai and coworkers (*50*).

S-Glutathionylation of proteins is an oxidative process; the oxidant is presumed to be traces of $O_2$ dissolved in the buffer during incubation. Oxidation of thiols yields disulfides, and formation of GSSG predominates when GSH is abundant. When GSH concentrations are low, the formation of protein–glutathione disulfides becomes more competitive. In contrast, when GSH was completely absent but HNE was present, only HNE–protein adducts were formed. The functional consequences of GST glutathionylation are not yet clear, but the formation of these adducts ex vivo under conditions of GSH depletion suggests that GSH depletion in vivo also leads to protein glutathionylation. If adducts are formed between proteins and both glutathione and reactive electrophiles, the contribution of each kind of modification toward enzyme inactivation deserves to be addressed. ESI mass spectra allow these modifications to be distinguished, and we are currently investigating the consequences of GST glutathionylation.

## Conclusions

Affinity purification using separate gradients of NaCl, GSH, and pH offers a fast (1 day) and simple method for isolation and purification of substantial quantities (~100 mg) of murine GST isozymes in greater than 90% purity as determined by HPLC. Short separation times avoid formation of oxidation or degradation products of GSTs that may occur during lengthy isolation procedures (*10*). This preparative method yielded high recoveries of intact GSTs with specific activities equivalent to those obtained with more time-consuming isolation procedures (*15–17*).

ESI/MS characterization of GST isozymes is fast (analysis time ~5 min) and straightforward and yields molecular masses with sufficient accuracy (within 2 Da) that minor differences in primary structure can be distinguished and agreements with cDNA sequences can be tested. Analysis of fractions from affinity purification allows for rapid assessment of isozyme identities and heterogeneity. ESI spectra also reveal whether modifications of GST isozymes occur during isolation, handling, or storage (e.g., formation of covalent adducts through disulfide bonds). It is conceivable that accidental modifications could be misinterpreted to be new GST isozymes based upon the appearance of new bands during electrophoretic or chromatographic separations.

Analysis by ESI/MS also provides clear evidence of covalent modification of GSTs due to either normal enzymatic processes (e.g., N-terminal acetylation of the alpha isozyme) or nonenzymatic processes including thiolation and covalent attachment of electrophiles. Such modifications are often difficult to recognize or quantify. The capabilities of ESI/MS are well suited to support studies of the role of covalent modification in altering protein function, and this approach can provide critical information regarding mechanisms of reversible or irreversible enzyme inhibition.

Traditional characterization of GST subunits is based upon measurements of p*I*, molecular mass (SDS–PAGE), and immunochemical recognition, but these techniques have limited abilities to resolve minor differences between known GSTs. Our review of the literature and sequence databases found more than a dozen reports of murine GST sequences with molecular masses that ranged from 23 478 to 25 970 Da. Given the numerous known forms of GSTs and the evidence of great genomic complexity for GSTs, it is recommended that routine characterization of GSTs by mass spectrometry be performed to clarify differences between GSTs obtained from different species, genders, strains, and tissues.

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