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Direct liquid chromatography–mass spectrometry method for the detection of glutathione *S*-transferase isozymes and investigation of their expression in response to dietary flavone

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Abstract

The cytosolic GSTs were measured directly in tissue homogenates using HPLC interfaced via ESI to a mass spectrometer (LC/MS). Total ion chromatograms were generated and filtered for ion currents corresponding to *m*/*z* ratio characteristic of individual GST isozymes. Direct LC/MS has a high degree of precision (8%) and low instrument detection limits (50–100 ng) and offers the advantage of monitoring GST expression at the protein level. In this study we describe the sub-chronic effect of feeding flavone (2500 mg/kg diet) on the expression of mGSTA3, mGSTP1, mGSTM1, and mGSTM2 in male and female mice. Additionally, we tentatively identify mGSTO and its up-regulation by flavone; a result that will facilitate the study of this novel enzyme. Flavone induced mGSTM1 and mGSTP1 in a gender and isozyme specific manner yet had no appreciable effect on the expression of mGSTA3. Male animals (day 5) displayed a 8-fold increase in mGSTM1 and a 2.6-fold increase in mGSTP1 whereas female animals displayed a 5- and 3-fold increase in mGSTM1 and mGSTP1, respectively. The mGSTM2 was detected only in flavone-fed animals, indicating an up-regulation of this isozyme by flavone. Results obtained using direct LC/MS compare favorably to the specific activity of individual isozymes ($P = 0.19$), and are comparable to GST levels determined using affinity chromatography followed by LC/MS ($P = 0.79$).

Keywords: Glutathione *S*-transferase isozymes

1. Introduction

Epidemiology studies strongly suggest a protective role for a diet rich in fruits and vegetables, yet mechanisms that afford this protection are not clear [1]. In particular, the intake of flavonoids is associated with a reduced risk of chronic diseases such as lung cancer [2] and coronary heart disease [3–6]. Biological activities of flavonoids include: potent antioxidant properties [7], the ability to induce apoptosis [8,9] and regulate the cell cycle [10,11]. The levels of specific flavonoids needed to produce effects in vitro are much higher than levels found in vivo [12], suggesting that secondary effects, such as the modulation of enzyme expression, are important factors in determining the in vivo activity of flavonoids [13]. Several studies indicate that certain flavonoid compounds can induce critical phase II biotransformation enzymes, such as glutathione *S*-transferases [14–16]. It is desirable to have rapid methods for screening the response of biotransformation enzymes in response to diet and environmental exposures.

Glutathione *S*-transferases (GSTs; EC 2.5.1.18) are the primary mammalian enzyme system responsible for detoxifying chemical carcinogens and by-products of lipid

Abbreviations: GSH, glutathione; GST, glutathione *S*-transferase; ESI, electrospray ionization; TIC, total ion chromatogram; RIC, reconstructed ion chromatograms; TFA, trifluoroacetic acid; CDNB, chloro-2,4-dinitrobenzene; BSP, bromosulfophthalein; DTT, dithiothreitol; HHM, horse heart myoglobin

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peroxidation [17]. Substrates for GSTs include aldehydic products of lipid peroxidation and carcinogenic products of phase I metabolism. Individual GST isozymes exhibit substrate specificity, and are expressed in a tissue-, gender-, species-, and age-related manner [17]. The three prevalent mammalian GST isozymes classes are alpha (mGSTA), mu (mGSTM), and pi (mGSTP) [18]. Other isozymes identified in mammals include sigma, theta, zeta and omega [19]. Within each class, multiple isoforms have been identified. Variation in GST isozyme expression can have profound effects on health. For example, low expression of human GSTM is associated with an increased incidence of bladder [20] and colon cancer [21]. Therapies that increase the expression of specific GST isozymes may prove useful as cancer prevention treatments.

A recently identified GST, termed omega (GSTO), demonstrates unusual catalytic and structural properties [19]. GSTO has low affinity for conjugating electrophiles with glutathione, and instead demonstrates activity similar to the glutaredoxins; including glutathione dependent dehydroascorbate reductase and thioltransferase activity [19]. GSTO has an active site cysteine that participates in the thioltransferase activity [19], and a hydrophobic site (H-site) that is more open and less hydrophobic then other GSTs. Consequently, GSTO may play a critical role in recovering oxidized proteins and peptides and restoring their function. The up-regulation of GSTO may be particularly important during oxidative stress.

In order to screen dietary compounds for their ability to modulate biotransformation reactions, a fast and reliable method is needed to quantitate levels of key enzymes such as the GSTs. The specific activity of individual GST isozymes is typically measured for this purpose. However, this is time consuming owing to the fact that independent assays with differing substrates are performed to monitor individual isozymes. Studies also routinely rely on the "universal" substrate 1-chloro-2,4-dinitrobenzene (CDNB) to measure total GST activity when in fact it is not a substrate for all isozymes (e.g. omega and theta). To address these issues, methods have been developed for the identification and quantitation of GST isozymes using glutathione-linked affinity chromatography followed by HPLC [22] and MS analysis [23–28]. Although these methods proved useful in the identification and quantitation of GSTs they required extensive sample preparation. Additionally, since GST isozymes are not equally retained on the glutathione-linked affinity matrix (e.g. theta and omega) not all isozymes are detected using this method. In addition, LC/ESI-MS/MS methods quantifying signature peptides of GSTA and GSTM after tryptic digestion have been developed [29], these quantitative methods are an improvement over other tryptic digest methods but they still require extensive sample preparation.

We report an improved LC/MS method, which requires little sample preparation prior to analysis, for the simultaneous analysis of GST isozymes directly in the cytosolic fraction of tissue homogenates. These methods represent a significant improvement in that they are fast, quantitative and represent expression at the protein level. New methods eliminate the use of the glutathione-linked affinity chromatography by taking advantage of orthogonal interface technologies and the ability to reconstruct ion chromatograms from the total ion current.

To validate the usefulness of this approach, a feeding study employing flavone (2500 mg/kg diet) over a period of 20 days was conducted in male and female Swiss Webster mice. Flavone was chosen as a model substrate since a previous study indicated that it was a potent inducer of mGSTM1 and mGSTP1 (Burns, unpublished). The results obtained using the direct LC/MS methods were comparable to specific activity, as well as to data obtained by affinity purification of individual isozymes followed by LC/MS quantitation, providing additional validation of this method.

2. Experimental

2.1. Materials

Flavone (97%) was purchased from Aldrich (Milwaukee, WI); trifluoroacetic acid (95%; TFA) in 1 ml ampules, was purchased from Acros (Pittsburgh, PA); 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid, glutathione reductase, tris(hydroxymethyl)aminomethane hydrochloride, cumene hydroperoxide (80%), horse heart myoglobin (HHM), and glutathione (GSH) agarose epoxy-linked through the sulfur were purchased from Sigma (St. Louis, MO), bromosulfophthalein (BSP) from ICN (Aurbra, OH). Bradford protein reagent was purchased from BioRad (Hercules, CA). All other reagents were analytical grade and purchased from Fisher (Pittsburgh, PA).

2.2. Animal treatment

Male (25–30 g) and female (18–20 g) Swiss Webster mice were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA). Male (four per group) and female (four per group) animals were housed in wire bottom cages and acclimated for one week prior to diet manipulation. Animals were fed ad libitum either a casein based control diet (AIN-76A, Dyets Inc) or the control diet with flavone incorporated at the concentration of 2500 mg flavone/kg diet. Animals were euthanized with carbon dioxide and the livers removed on days 5, 10 or 20.

2.3. Sample preparation

The four livers from each group were combined, homogenized and the cytosolic fraction isolated using previously described methods [24]. The resulting supernatant was collected and specific activity was measured following centrifugation. The remaining supernatant was frozen at −80 °C until analysis by LC/MS. Frozen supernatants were defrosted on

Table 1
The molecular masses, linear regression equations, and correlation coefficients (R^2) for the GST external standards and the ions selected to quantify the levels of GST isozymes in murine hepatic tissue

Isozyme	MW	Linear regression equation	R^2	Ions used for GST isozyme identification											
mGSTA3	25272	$y = 255351x + 1611799$	0.98	1204.4	1149.7	1099.7	1054.0	1011.8	973.0	937.0	903.5	872.4	843.4	816.2	790.7
mGSTP1	23478	$y = 227062x + 627886$	0.99	1236.7	1174.9	1119.0	1068.2	1021.8	979.3	940.1	904.0	870.6	839.5	810.6	783.6
mGSTM1	25838	$y = 306411x + 2891270$	0.98	1175.9	1124.8	1078.0	1034.9	995.2	958.3	924.1	892.3	862.6	834.8	808.8	784.3
mGSTM2	25580	NA[a]		1113.2	1024.2	984.8	948.4								
mGSTO	27195	NA[a]		938.8	907.5	878.3	850.8								

[a] The levels of mGTSM2 and mGST0 were quantified using mGSTM1 as an external standard.

ice and diluted (1:20) with nanopure water prior to LC/MS analysis.

For comparison purposes, aliquots from three of the samples were also analyzed following established methods employing glutathione affinity purification prior to LC/MS analysis [24].

2.4. Liquid chromatography

HPLC separations were performed using a Shimadzu (Kyoto, Japan) SCL-10AVP system. Chromatography was achieved using a Supelco (Bellefonte, PA) Discovery BIO wide pore C_{18} column (25 cm × 2.1 mm, 5 μm). Mobile phases consisted of 0.05% TFA in water (A) and 0.05% TFA in ACN (B). The initial condition of 40% B was held for 1 min. GST proteins were separated using a linear gradient from 40 to 45% B in 7 min, followed by a gradient of 45–55% B in 17 min at a flow rate of 0.2 ml/min.

2.5. ESI/mass spectrometry

The proteins were detected using a Quattro LC triple quadrupole mass spectrometer (Micromass, Altrincham, UK) equipped with a Z-Spray electrospray (ESI) interface. Ionization conditions were optimized using purified GST (mGSTA3, mGSTP1 and mGSTM1) standards. The ion source temperature was maintained at 150 °C and the desolvation gas temperature at 300 °C. The ESI was operated in positive ion mode with a cone voltage of 35 V, capillary voltage of 3 kV and extractor voltage of 2 V. Nitrogen was used as the drying gas at 800 L/h and as the cone gas at 48 L/h. The analysis was performed by collecting the TIC in continuum mode over a mass range of *m*/*z* 600 to 1400 with a cycle time of 2.1 s, a scan duration of 2.0 s and an inter-scan delay of 0.1 s.

2.6. GST standards

GST isozymes mGSTM1, mGSTP1 and mGSTA3 were purified using previously described methods [23]. Isozymes were determined to be >95% pure by mass spectrometry. Purified GST isozymes were accurately weighed, then combined to produce a standard of known concentration; dilutions of this stock solution were made to produce the standard curves over a linear range of 100–5000 ng. Instrument detection limits for these LC/MS methods are 50 ng for mGSTP1 and mGSTM1 and 100 ng for mGSTA3. Regression equations and the correlation coefficients for these curves are given in Table 1. Protein molecular masses were determined using MaxEnt Masslynx software (version 3.5; Micromass, Wythenshawe, UK) and are listed in Table 1. Horse heart myoglobin was used to calibrate the mass spectrometer.

2.7. Quantification

TICs were filtered, by selecting the most abundant ions characteristic for mGSTM1, mGSTM2, mGSTA3, mGSTP1 and mGSTO (Table 1), creating RICs. Fig. 1 is a representative mass spectrum of mGSTM illustrating the charge distribution and ions chosen for peak identification. Identifying ions were chosen by picking the four most abundant ions in the charge envelope (±0.5 amu) corresponding to calculated ion masses based on the known protein sequence. Four consecutive ions on either side of this distribution were also included to account for variable charge distributions. In these experiments all mass spectra were obtained under identical ionization conditions minimizing variation in the charge distribution. In the case of mGSTO and mGSTM2 only the four most abundant ions apparent in each spectrum were used to obtain RIC. Peak areas obtained from the RIC were used to quantitate proteins (Fig. 2). In contrast, the samples treated by GSH affinity purification prior to LC/MS were quantified by integrating the TICs. The concentrations of mGSTO and mGSTM2 were estimated using mGSTM1 as an external standard. Samples and standards were analyzed in triplicate. The method detection limit was determined by adding horse

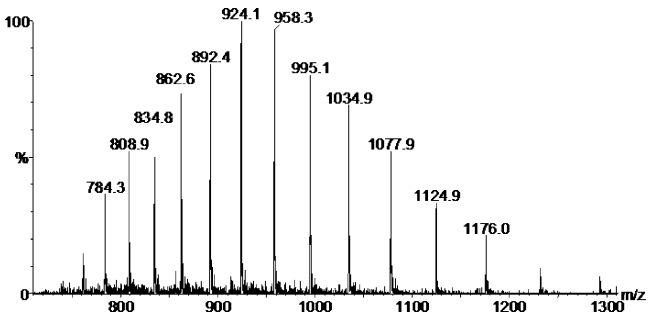

Fig. 1. Typical mass spectra of purified mGSTM1obtained from ESI/MS analysis. The mass spectrometer operated in full scan mode, the total ion chromatogram (TIC) was collected over a range of *m*/*z* 600–1400.

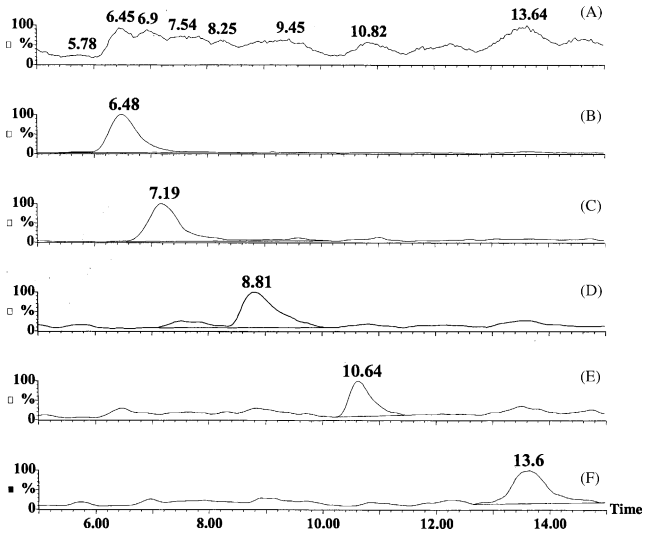

Fig. 2. The LC/MS total ion chromatogram (TIC) of GST isozymes and reconstructed ion chromatograms (RIC) filtered for ions specific to mGSTM1, mGSTM2, mGSTA3, mGSTP1, and mGSTO. The cytosolic fraction of liver homogenates were analyzed by the mass spectrometer operated in full scan mode, the TIC was collected over a range of *m/z* 600–1400 (A), RICs were generated by selecting ion currents from characteristic of mGSTM1 (B), mGSTM2 (C), mGSTA3 (D), mGSTP1 (E), and mGSTO (F).

heart myoglobin (HHM) to the diluted cytosolic fraction of liver homogenate prior to LC/MS analysis.

Protein modification due to oxidation in the presence of GSH often results in glutathionylation, in fact this modification occurs as a common artifact of GST purification [23]. In this study, the purified mGSTA3 standard was determined to be 44% glutathionylated. Conversely, GSTs analyzed directly in tissue homogenates display no glutathionylation indicating that glutathionylation occurs during affinity purification in glutathione-rich buffers. Therefore, mGSTA3 values in the samples were corrected to account for this overestimation. No other oxidative artifacts were detected.

2.8. Statistical analysis

The coefficient of variation was used to measure precision using replicate injections of the samples ($N = 3$). Paired Student's *t*-tests (two-tailed, homoscedastic) were applied to data comparing affinity LC/MS method and specific activity assays to this direct LC/MS method.

2.9. GST enzyme activity

GST activity was measured at 25 °C using CDNB as a substrate to determine total GST activity [30]. BSP, ethacrynic acid and GSH peroxidase were used to determine mGSTM, mGSTP, and mGSTA activity, respectively [30,31]. The Bradford method was used to determine protein concentration [32].

2.10. Western blot

The eluant from the HPLC containing protein with a MW corresponding to mGSTO was collected. Approximately 100 μg protein was separated on a 10% SDS–PAGE gel. Western blots were developed using rabbit antiserum raised against His-tagged recombinant GSTO 1-1 and goat anti-rabbit IgG alkaline phosphatase conjugate [19].

3. Results and discussion

In the current study the use of LC/MS to investigate levels of GST isozymes expressed directly in tissue homogenates was explored. Our results indicate that this approach is both a fast and reliable method for the quantification of GST expression in tissue samples and values obtained compare favorable with values obtained using lengthier purification methods. The methods reported here have a high level of mass accuracy (1–2 Da) and precision (8.0%). The method detection limit was determined to be 4 μg/mg protein. To validate this new approach the levels of the three most prevalent GST isozymes (i.e. mGSTA3, mGSTM1, and mGSTP1) were measured in hepatic tissue of male and female Swiss Webster mice following dietary exposure to flavone (2500 mg/kg diet) for 5, 10 or 20 days. In previous studies we found that flavone was a potent inducer of GSTs (Burns unpublished). Levels of mGSTA3, mGSTM1, and mGSTP1 were easily measured in both treated and untreated animals, whereas mGSTM2 isozyme (a minor isozyme) was detectable in only in the flavone-fed animals indicating that it was likely up-regulated by flavone.

Levels of GST expression measured using direct LC/MS were compared with the specific activity of mGSTA3, mGSTP1 and mGSTM1 in these samples (Table 2). The results obtained by each method were equivalent ($P = 0.19$; high P values indicate similar results) although estimates of GST induction were generally higher when measured by specific

Table 2
A comparison of hepatic GST expression measured directly in cytosol by LC/MS (μg/mg protein) and by measuring specific acitivity (μmol/min mg protein) following dietary exposure to flavone (2500 mg/kg diet). Reported values represent GST induction as they are presented as a ratio of treated:control

Isozyme	Gender	Specific activity			LC/MS		
		5 day	10 day	20 day	5 day	10 day	20 day
mGSTA3	M	0.95	0.82	0.75	1.93	0.97	0.89
	F	0.64	0.78	0.73	0.91	0.93	0.89
mGSTM1	M	6.97	7.34	4.01	8.37	3.81	4.14
	F	10.27	5.62	8.46	5.24	6.61	5.85
mGSTP1	M	2.08	1.37	1.13	2.58	1.24	1.13
	F	4.15	3.79	3.78	3.15	2.70	2.36
Total GST	M	5.19	4.19	3.60	4.03	1.89	1.92
	F	5.90	7.30	6.46	2.57	2.97	2.67

Table 3
Concentrations of mGSTA3, mGSTP1 and mGSTM1 in three samples determined by direct LC/MS analysis compared to concentrations determined using affinity purification prior to LC/MS analysis

Sample ID	GST isozyme	Affinity chromatography prior to LC/MS (μg/mg protein)	Direct LC/MS (μg/mg protein)
Control female day 20	mGSTP1	8.75	9.47
	mGSTM1	18.98	20.27
	mGSTA3	11.38	22.94
Control female day 10	mGSTP1	10.75	9.51
	mGSTM1	21.26	19.63
	mGSTA3	20.85	20.83
Control male day 5	mGSTP1	16.44	17.87
	mGSTM1	29.84	27.73
	mGSTA3	21.62	18.51

activity. It is important to note that although enzyme activity is a good estimate of protein expression, the response can deviate from linearity due to protein oxidation and alterations in cellular GSH levels [33]. This is particularly a problem when monitoring dietary regulation of GSTs since certain flavonoids can increase intracellular GSH levels through the induction of γ-glutamylcysteine synthetase [34]. Additional problems may arise when using CDNB to measure GST expression since certain GST isozymes (e.g. theta and omega) display little to no activity towards CDNB.

To further validate the approach of measuring GSTs directly in the cytosol by LC/MS, an aliquot of the cytosol was taken from a subset of samples and analyzed according to methods employing affinity chromatography followed by LC/MS [24]. The affinity chromatography method has greater than 80% recoveries when measured by losses in specific activity [24]. In general, results obtained using affinity purification prior to LC/MS analysis were equivalent (P = 0.79) to results obtained by direct LC/MS analysis of GSTs in tissues homogentates (Table 3). An exception was found in one sample analyzed by the combined affinity purification and LC/MS method. In this sample we found a 50% decrease in mGSTA3. A corresponding decrease in GST activity occurred during the affinity purification step, which indicates that a loss of protein occurred during the affinity purification. In contrast, the direct analysis of GSTS in tissue homogenates is not subject to protein losses that can occur during affinity purification.

This method was utilized to determine the impact of dietary flavone on hepatic GST expression. Results indicate that dietary flavone influences GST expression in a gender- and isozyme-specific manner (Table 2) with the greatest impact on mGSTM1. Constitutively expressed (control) levels of mGSTM1 were similar in male (25.35 μg/mg protein) and female (22.86 μg/mg protein) animals. In response to flavone, male animals demonstrated a peak in mGSTM1 levels, corresponding to an eight-fold induction, on day 5 (Fig. 3). These levels remained elevated throughout the 20-day trial (four-fold induction on day 20). In female animals fed flavone, levels of mGSTM1 were consistently elevated throughout the 20 days and ranged between a five- and seven-fold induction.

The levels of mGSTM2 in control animals were below the method detection limit of 4 μg/mg protein whereas they were easily measured in flavone-fed animals. This is not surprising as levels of mGSTM2 in this mouse strain were previously determined to be approximately 9% of the concentration of mGSTM1 [25] which corresponds to approximately 2 μg/mg protein. The levels of mGSTM2 in flavone-fed female animals ranged from 20.19 μg/mg protein (day 5) to 17.39 μg/mg protein (day 20). In flavone-fed males, mGSTM2 levels ranged from 25.43 μg/mg protein (day 5) to 15.16 μg/mg protein (day 20). The levels in flavone-fed animals are approximately 10-fold higher than estimated control levels suggesting that dietary flavone up-regulates the expression of this GST isozyme.

Dietary flavone had a greater influence on mGSTP1 expression in female animals as compared to male animals. Constitutively expressed levels of mGSTP1 were higher in male (28.3 μg/mg protein) animals as compared to female (8.40 μg/mg protein) animals (Fig. 4A and B). In response to flavone, male animals demonstrated a 2.6-fold induction of mGSTP1 on day 5. No induction was noted on days 10

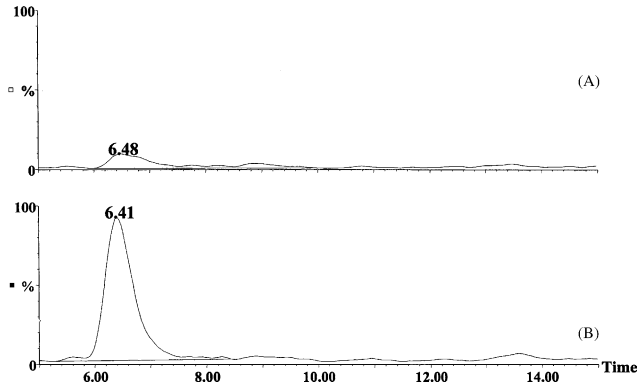

Fig. 3. Reconstructed ion chromatogram (RIC) of mGSTM1 in male mice following dietary exposure to flavone (2500 mg/kg diet) for 5 days. The cytosolic fraction of liver homogenates were analyzed by the mass spectrometer operated in full scan mode, the total ion chromatogram (TIC) was collected over a range of m/z 600–1400. RICs were generated by filtering the TIC for the ions specific to mGSTM1 from control males (A) and flavone-fed males (B) on day 5.

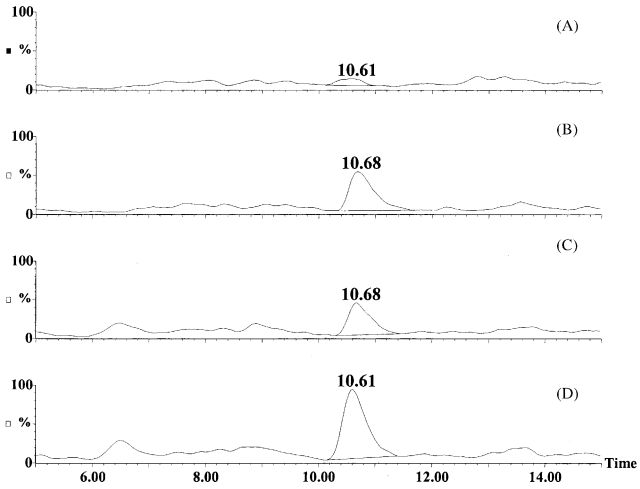

Fig. 4. Reconstructed ion chromatogram (RIC) of mGSTP1 in male and female mice following dietary exposure to flavone (2500 mg/kg diet) for 20 days. The cytosolic fraction of liver homogenates were analyzed by the mass spectrometer operated in full scan mode, the total ion chromatogram (TIC) was collected over a range of *m*/*z* 600–1400. RICs were generated by filtering the TIC for the ions specific to mGSTP1in control females (A), control males (B), flavone-fed females (C) and flavone-fed males (D).

or 20. Conversely, female animals displayed an approximate 2–3-fold induction throughout the 20-day study with levels peaking on day 10 of the study (Fig. 4C and D).

Dietary flavone only influenced the expression of mGSTA3 in male animals. Constitutively expressed levels of mGSTA3 were similar in male (18.58 μg/mg protein) and female (22.11 μg/mg protein) animals. A 1.9-fold induction of mGSTA3 occurred in male animals on day 5. These levels returned to constitutively expressed levels by day 10. No change in mGSTA3 was observed in female animals.

The levels of GSTs we report are similar to values obtained for human hGSTA1 and hGSTM1 (these ranged from 17–53 to 6–14 μg/mg, respectively) through the quantification of signature peptides of GSTA and GSTM after tryptic digestion [29].

Similar levels of hGSTA1 in human intestinal 6.6 μg/mg protein [35] and pancreatic tissues 1.4 μg/mg protein [36] also have been reported.

During these studies we noticed the induction of a GST-like protein with a mass similar to the calculated molecular mass of murine GST omega (mGSTO; 27,497 Da) and to GSTO purified from pig liver (27,328 Da). Western blot analysis of HPLC fractions corresponding to this protein indicates that it belongs to the GSTO class of GSTs. Constitutively expressed levels of mGSTO were similar in male and female animals (17.5 μg/mg protein). Interestingly, dietary flavone produced a transient induction of mGSTO that peaked on day 5 (1.8-fold in male animals and 1.4-fold in female animals). The identification and quantification of mGSTO has been hindered in the past due to its low affinity to GSH affinity matrixes and lack of activity towards CDNB. The identification of this protein in murine liver tissue and the discovery of an inducer will aid in the characterization of this enzyme.

Direct analysis of GSTs in tissue homogenates using LC/MS provides investigators with a rapid screening tool for monitoring isozyme specific expression of GSTs, at the protein level, in response to a variety of environmental and dietary exposures. Given that many common dietary flavonoids induce GST activity, and induction of GSTP is considered protective against the development of cancer, it is critical to develop rapid and reliable methods for screening large numbers of flavonoids for this activity.

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References

[1] B.N. Ames, L.S. Gold, Drug Metab. Rev. 30 (1998) 201.
[2] P. Knekt, R. Jarvinen, R. Seppanen, M. Hellovaara, L. Teppo, E. Pukkala, A. Aromaa, Am. J. Epidemiol. 146 (1997) 223.
[3] M.G. Hertog, E.J. Feskens, P.C. Hollman, M.B. Katan, D. Kromhout, Lancet 342 (1993) 1007.
[4] M.G. Hertog, E.J. Feskens, D. Kromhout, Lancet 349 (1997) 699.
[5] P. Knekt, R. Jarvinen, A. Reunanen, J. Maatela, BMJ 312 (1996) 478.
[6] L. Yochum, L.H. Kushi, K. Meyer, A.R. Folsom, Am. J. Epidemiol. 149 (1999) 943.
[7] C. Rice-Evans, Curr. Med.Chem. 8 (2001) 797.
[8] S. Ueda, H. Nakamura, H. Masutani, T. Sasada, A. Takabayashi, Y. Yamaoka, J. Yodoi, Mol. Immunol. 38 (2002) 781.
[9] U. Wenzel, S. Kuntz, M.D. Brendel, H. Daniel, Cancer Res. 60 (2000) 3823.
[10] W. Wang, L. Heideman, C.S. Chung, J.C. Pelling, K.J. Koehler, D.F. Birt, Mol. Carcinog. 28 (2000) 102.
[11] T. Kobayashi, T. Nakata, T. Kuzumaki, Cancer Lett. 176 (2002) 17.
[12] P.C.H. Hollman, J. Sci. Food Agric. 81 (2001) 842.
[13] D.F. Birt, S. Hendrich, W. Wang, Pharmacol. Ther. 90 (2001) 157.
[14] V. Breinholt, S.T. Lauridsen, L.O. Dragsted, Xenobiotica 29 (1999) 1227.
[15] M.C. Canivenc-Lavier, M.F. Vernevaut, M. Totis, M.H. Siess, J. Magdalou, M. Suschetet, Toxicology 114 (1996) 19.
[16] W.A. Nijhoff, M.A. Bosboom, M.H. Smidt, W.H. Peters, Carcinogenesis 16 (1995) 607.
[17] J.D. Hayes, D.J. Pulford, Crit. Rev. Biochem. Mol. Biol. 30 (1995) 445.
[18] M. Warholm, H. Jensson, M.K. Tahir, B. Mannervik, Biochemistry 25 (1986) 4119.
[19] P.G. Board, M. Coggan, G. Chelvanayagam, S. Easteal, L.S. Jermiin, G.K. Schulte, D.E. Danley, L.R. Hoth, M.C. Griffor, A.V. Kamath, M.H. Rosner, B.A. Chrunyk, D.E. Perregaux, C.A. Gabel, K.F. Geoghegan, J. Pandit, J. Biol. Chem. 275 (2000) 24798.
[20] A.K. Daly, D.J. Thomas, J. Cooper, W.R. Pearson, D.E. Neal, J.R. Idle, BMJ 307 (1993) 481.
[21] R.C. Strange, B. Matharoo, G.C. Faulder, P. Jones, W. Cotton, J.B. Elder, M. Deakin, Carcinogenesis 12 (1991) 25.
[22] A.K. Ostlund Farrants, D.J. Meyer, B. Coles, C. Southan, A. Aitken, P.J. Johnson, B. Ketterer, Biochem. J. 245 (1987) 423.

[23] A.E. Mitchell, D. Morin, M.W. Lame, A.D. Jones, Chem. Res. Toxicol. 8 (1995) 1054.
[24] A.E. Mitchell, D. Morin, J. Lakritz, A.D. Jones, Biochem. J. 325 (Pt. 1) (1997) 207.
[25] A.E. Mitchell, J. Lakritz, A.D. Jones, Arch. Toxicol. 74 (2000) 215.
[26] S. Treptow-van Lishaut, G. Rechkemmer, I. Rowland, P. Dolara, B.L. Pool-Zobel, Eur. J. Nutr. 38 (1999) 76.
[27] P. Rouimi, L. Debrauwer, J. Tulliez, Anal. Biochem. 229 (1995) 304.
[28] J.D. Rowe, E. Nieves, I. Listowsky, Biochem. J. 325 (Pt. 2) (1997) 481.
[29] F. Zhang, M.J. Bartels, W.T. Stott, Rapid Commun. Mass Spectrom. 18 (2004) 491.
[30] W.H. Habig, M.J. Pabst, W.B. Jakoby, J. Biol. Chem. 249 (1974) 7130.
[31] D.E. Paglia, W.N. Valentine, J. Lab. Clin. Med. 70 (1967) 158.
[32] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[33] S.-T. Wang, H.-W. Chen, L.-Y. Sheen, C.-K. Lii, J. Nutr. 127 (1997) 2135.
[34] M.C. Myhrstad, H. Carlsen, O. Nordstrom, R. Blomhoff, J.O. Moskaug, Free Radic. Biol. Med. 32 (2002) 386.
[35] B.F. Coles, K.E. Anderson, D.R. Doerge, M.I. Churchwell, N.P. Lang, F.F. Kadlubar, Cancer Res. 60 (2000) 573.
[36] B.F. Coles, G. Chen, F.F. Kadlubar, A. Radominska-Pandya, Arch. Biochem. Biophys. 403 (2002) 270.