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Rapid method for the determination of glutathione transferase isoenzymes in crude extracts

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ABSTRACT

The analysis of glutathione transferase (GST) isoenzyme patterns is of interest in many fields as hepatic glutathione transferase activity is increased by exposure to a variety of xenobiotics and its isoenzymatic forms are induced differentially. A high-performance liquid chromatography method has been developed for the rapid determination of individual isoenzyme levels in crude extracts using an anion-exchange column connected to an on-line system to automatically detect GST activity with 1-chloro-2,4-dinitrobenzene as the substrate. When 50–200 μl of a cytosolic fraction of fish liver containing up to 15 mg/ml of protein and less than 2 units of GST were injected, a high resolution and highly reproducible chromatogram was obtained. The activity profile determined automatically showed eight to twelve peaks (depending on the sample) that were quantified and could be classified into three groups. Starting from intact tissue, a complete isoenzyme pattern could be obtained in less than 3 h. The method has been applied to ecotoxicological studies with fish samples.

INTRODUCTION

Glutathione transferases (GSTs) are a widespread family of isoenzymes implicated in the detoxification of electrophilic xenobiotics as well as in several endogenous functions. One isoform is membrane-bound [1] and another is nuclear [2]. Cytosolic GST isoenzymes are dimers of a number of subunits with molecular masses of about 25 000 and have been grouped into four classes with respect to physiological, structural and genetic similarities: α, μ, π and θ [3,4]. 1-Chloro-2,4-dinitrobenzene (CDNB) is used as a universal substrate for GST isoenzymes.

A wide variety of structurally unrelated compounds have been shown to increase hepatic GST activity in a number of organisms, mostly in laboratory animals (mice and rats). Differential induction of GST isoenzymes has been reported [5,6]. Induction of a particular isoform by a given compound can be masked if only the overall GST activity of a crude extract is analysed, hence the whole isoenzyme pattern needs to be determined. To this end, several methods have been devised which include an initial affinity chromatography step on GSH–Sepharose or *S*-hexylgluirring (500 rpm). The levels of peroxyacids and hydrogen peroxide were expressed as the mean value of five repeated experiments.

Chromatographic apparatus and conditions

The chromatographic apparatus consisted of an LC-6A HPLC pump (Shimadzu, Japan) equipped with an additional external pulse damper (Shimadzu) and a Type 7125 injection valve (Rheodyne, Cotati, CA, USA). Solvents were degassed using a Type ERC-3110 degasser (Erma Optical Work, Japan). A LiChrosorb RP-18 (10 μm do not bind or bind loosely lowering recovery yields [6,9,12].

This paper reports a single-step analytical method based on ion-exchange HPLC with automatic on-line detection of activity for the rapid determination of GST isoenzymes in crude biological samples, using CDNB as a non-specific substrate.

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MATERIALS AND METHODS

Chemicals

CDNB and salts for the mobile phases were from Merck; bovine serum albumin (BSA), reduced glutathione (GSH), dithiothreitol (DTT), hexylglutathione, phenylmethylsulphonylfluoride (PMSF) and *S*-hexylglutathione-agarose were from Sigma. HPLC-grade water was obtained with a Milli-Q system (Millipore). All chemicals used were of analytical-reagent grade.

Buffers and reagents

Buffers were prepared at working temperatures (4°C and room temperature). The extraction buffer contained 10 m*M* Tris–HCl (pH 7.5), 1 m*M* EDTA, 5 m*M* GSH, 2 m*M* DTT and PMSF at a final concentration of 0.5 mg/g of tissue. The loading buffer (A in Fig. 1) contained 10 m*M* Tris–HCl (pH 8.0), 0.2 m*M* EDTA and 0.2 m*M* DTT; the salt buffer (B) was the same as the loading buffer supplemented with 0.5 *M* NaCl. To prepare 100 ml of reagent C the following solutions were mixed at room temperature in the order indicated: 30 ml of 13.3 m*M* CDNB in ethanol; 42 ml of water; 25 ml of 0.4 *M* sodium phosphate, pH 6.5; 1 ml of 0.1 *M* EDTA; 2 ml of 10 mg/ml BSA. The same procedure was followed to prepare 100 ml of reagent D except that CDNB was substituted for 30 ml of ethanol and 0.121 g of crystalline GSH was dissolved in the final solution. Solutions C and D were sonicated. All solutions were filtered through a 0.45-μm Millipore membrane and degassed.

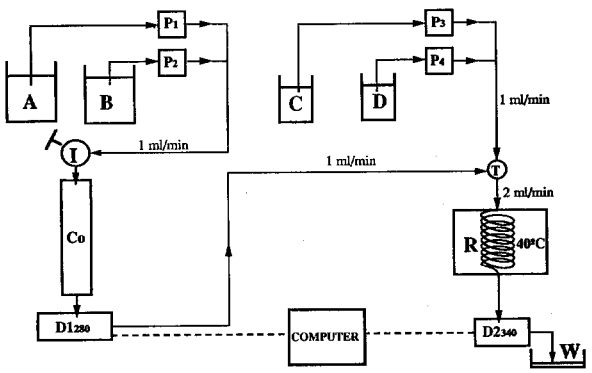

Fig. 1. Schematic diagram of the experimental set up. A = Loading buffer; B = elution buffer; C = CDNB solution; D = GSH solution; P1 and P2 = buffer pumps; P3 and P4 = reagent pumps; I = sample injector: Co = anion-exchange chromatographic column; $D1_{280}$ = protein detector set at 280 nm; $D2_{340}$ = activity detector set at 340 nm; T = T union; R = reactor coil in a water-bath at 40°C; W = waste.

Chromatographic arrangement

A schematic description of the HPLC set-up is presented in Fig. 1. P4 and D2 were a pump from a Series 2 liquid chromatograph and LC-55 B spectrophotometer, respectively, both from Perkin-Elmer. The rest of the equipment was from Beckman Instruments unless stated otherwise. P1, P2 and P3 were 110B pumps; D1 was a 263 UV variable-wavelength detector; the system was controlled by a 406 analog interface module; data collection and processing was carried out by an AT computer and System Gold software. The column was a DEAE-Spherogel TSK, 10 μm particle size (7.5 × 0.75 cm). Extracts were passed through a 0.45-μm Millipore filter before injection into the column. A precolumn was used to avoid clogging and contamination of the analytical column.

Preparation of crude extracts and pure GST

Livers from the marine fish *Mugil* sp. were obtained immediately after capture and frozen in liquid nitrogen. They were ground in a mortar while immersed in the same coolant. The powder was kept at −80°C and, when needed, was suspended in extraction buffer (4 ml/g) and disrupted using an Ultra Turrax homogenizer. The resulting homogenate was centrifuged at 31 000 *g* for 30 min at 4°C in a J2-21 Beckman centrifuge. The supernatant was further cleared by ultracentrifugation at 105 000 *g* for 1 h at 4°C in a Beckman L8-80M apparatus. The first centrifugation step was occasionally omitted. In some experiments GST was further purified by affinity chromatography in a column (6 × 0.5 cm) of hexylglutathione–agarose equilibrated in 50 m*M* phosphate buffer (pH 7.0), 1 m*M* EDTA and 2 m*M* DTT. The column was washed with 50 m*M* KCl in the same buffer and eluted with a pulse of 5 m*M* hexylglutathione in 50 m*M* Tris–HCl buffer (pH 9.6). The pooled fractions were concentrated by vacuum evaporation and dialysed against extraction buffer without PMSF.

Assays

GST activity was assayed at 37°C following the method of Habig and Jakoby [13] using 1 m*M*

CDNB and 1 m*M* GSH. When dilute enzyme preparations were assayed, 0.1 mg/ml BSA was included in the reaction mixture. The protein concentration was determined by the method of Lowry *et al.* [14] using BSA as the standard.

RESULTS AND DISCUSSION

System design and optimization of reaction conditions

Solutions C and D, containing CDNB and GSH, respectively (Fig. 1) were intended to have the same viscosity to allow for good mixing, thus giving a low-noise baseline. This is particularly important if only one reagent pump is available. Ethanol was needed to dissolve CDNB, but its concentration was compromised to allow good solubility of CDNB without reaching a limit above which phosphate might crystallize. The final composition of the assay mixture in the reactor was 15% (v/v) ethanol, 1 m*M* CDNB, 50 m*M* phosphate (pH 6.5), 0.5 m*M* EDTA, 0.1 mg/ml BSA and 1 m*M* GSH. With a 5 m long reactor the reaction time was 0.5 min. The effect of 15% ethanol on the enzymatic activity under the standard assay conditions was to decrease the activity by 11%, which was considered acceptable.

Initially, the response of the system was checked without the column. There was an excellent linearity between the amount of enzyme injected and the peak area. The peaks were symmetrical and their retention times, that is from the time the signal starts until the baseline value is reached again, were always between 1.6 and 1.9 min for sample volumes greater than 25 μl. This was taken as an indication that mixing and flow through the coils were good.

The set-up of Fig. 1 without the column can be used as a spectrophotometric flow injection analysis (FIA) system. This application could be of value as it would allow the analysis of about 30 samples/h. Optimization would be completed by an automatic sample injector.

Chromatographic behaviour

Crude extracts from fish livers were run on the system equipped with a DEAE-Spherogel column equilibrated in loading buffer and eluted with a linear NaCl gradient (0–0.35 *M*) starting at 15 min and ending at 60 min. The separation was excellent and repetitive a judged either from the A_{280} profile for total protein or from the A_{340} profile for GST

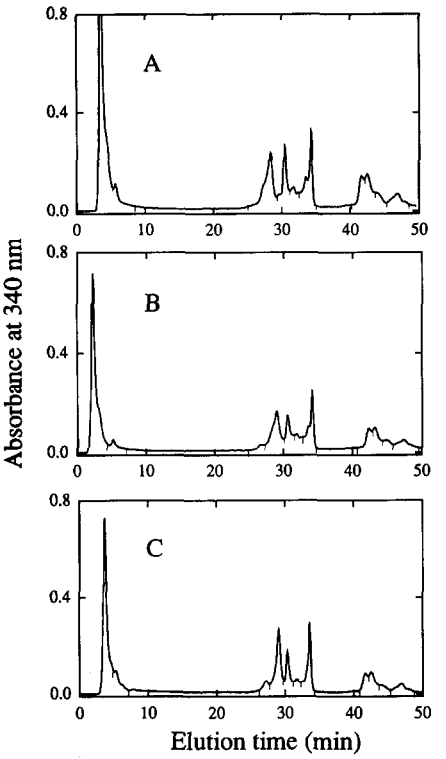

Fig. 2. GST activity profiles of an extract from fish liver. The sample was the soluble fraction from a pool of fish (*Mugil* sp.) livers with 16.0 U/ml GST. Volumes injected were 50 μl of undiluted preparation in A and 100 μl of a 1:3 dilution in B and C. The small downward vertical lines are integration marks indicating the beginning and end of each peak. See text for other details of the procedure.

activity. Variation of sample volume in the range 50–200 μl, containing up to 15 mg/ml protein, did not affect separation, but too much activity in the sample saturated the detection system. Hence the GST activity of the extract was previously determined to define the amount of sample to be injected and this should not be higher than 2 units. If necessary, the sample was conveniently diluted in extraction buffer immediately before injection. Another way to circumvent the problem of saturating the detection set-up could be to shorten the length of the reactor or to lower its temperature, but we preferred to keep these factors unchanged to maintain the standard conditions of the whole arrangement. Alernatively, the length of the reactor could be increased to detect the isoenzyme pattern of samples with low GST activity towards CDNB (*e.g.*,

TABLE I

DETERMINATION OF INDIVIDUAL ISOENZYME CONTENTS

The data presented are averages of the three chromatograms shown in Fig. 2.

Peak No.	Retention time (mean ± S.D.) (min)	Peak area[a]	
		Mean ± S.D. (U/μl)	Relative standard deviation (%)
1	3.50 ± 0.17	17.77 ± 1.90	11
2	5.68 ± 0.36	1.73 ± 0.51	29
3[b]	n.d.	n.d.	n.d.
4	28.8 ± 0.32	6.64 ± 0.60	9
5	30.4 ± 0.09	3.40 ± 0.32	9
6	31.7 ± 0.09	1.38 ± 0.13	9
7	34.0 ± 0.30	5.47 ± 0.04	1
8	41.9 ± 0.29	1.87 ± 0.03	2
9	42.7 ± 0.32	2.80 ± 0.11	4
10	44.2 ± 0.44	0.97 ± 0.07	7
11	47.0 ± 0.22	1.71 ± 0.08	5
Total		43.74 ± 1.64	4

[a] One arbitrary unit of peak area corresponds to 0.367 ± 0.017 mUnits of GST activity. Peak area values have been divided by the volume of undiluted preparation injected, that is, 50 μl for A and 33.3 μl for B and C.

[b] Peak No. 3 was not clearly separated in chromatogram A and for that reason was considered as part of peak 4 in all three chromatograms when calculating average peak areas. n.d. = Not determined.

< 2 units/ml). However, this situation did not arise during this study.

Fig. 2 shows the activity profiles obtained when different volumes, 50 μl in A and 100 μl of a 1:3 dilution of the same sample in B and C, were injected. The repeatability of the method is demonstrated by the striking coincidence of the chromatograms. The results of integrating these chromatograms are summarized in Table I. Depending on the sample, between eight and twelve peaks were resolved and were numbered according to their order of elution from the column. Previously reported anion-exchange HPLC of GST from human placenta resolved just three peaks [15], whereas Foureman and Bend [16] separated five peaks of GST from the liver of skate on a peparative DEAE–cellulose column. Lee *et al.* [17] also separated five peaks on a DEAE–cellulose column from the anionic forms of rat liver.

The isoenzymes could be classified into three groups. Group I was composed of the isoforms that either passed through the column or were retarded but not adsorbed. Two isoenzymes were resolved in this instance. A prominent shoulder around 4.5 min was considered as part of the first peak, although many other samples gave three well separated peaks in this zone, whereas in conventional low-pressure DEAE–Sepharose chromatography they co-elute [18,19]. Theoretically, these are the basic and neutral species comparable with those from other organisms. However, with ion-exchange chromatography apparently contradictory results can be obtained. For instance, Kispert *et al.* [20] found that "neutral" GST-μ from rat testes behaves as a true acidic form as it binds to a Mono-Q (anionic) column at pH 8.0 requiring 0.15 *M* NaCl for elution. Hence the order of elution from an ion-exchange chromatographic column cannot be used with confidence to classify the isoenzymes according to their acid–base or electrical properties. Other non-ionic interactions taking place between the exchanger matrix and the proteins have marked effects on their chromatographic behaviour.

Among the isoenzymes retained in the column, those interacting more weakly eluted before 35 min and were included in group II. Acidic isoenzymes that were more tightly bound to the column belonged to group III and were eluted with higher ionic strength (later than 35 min).

The data of Table I show how reproducible the method is, with the variability of peak elution times never exceeding 30 s, that is, less than 1% of the total elution time. The table also shows a very good proportionality between volume of sample injected and peak area (both total and individual). The highest deviations were found among isoenzymes of group I considered individually, although taken altogether their average total area per microlitre injected was 19.51 ± 1.75, that is, a relative standard deviation of 9%.

When affinity purified GST preparations were run and protein profiles were matched with activity profiles, it was observed that isoenzymes of group II were those with the highest specific activity, whereas those eluting in group III had the lowest specific activity. Occasionally the column eluate was collected in fractions of 300 μl to determine the activity manually. The chromatographic activity profile

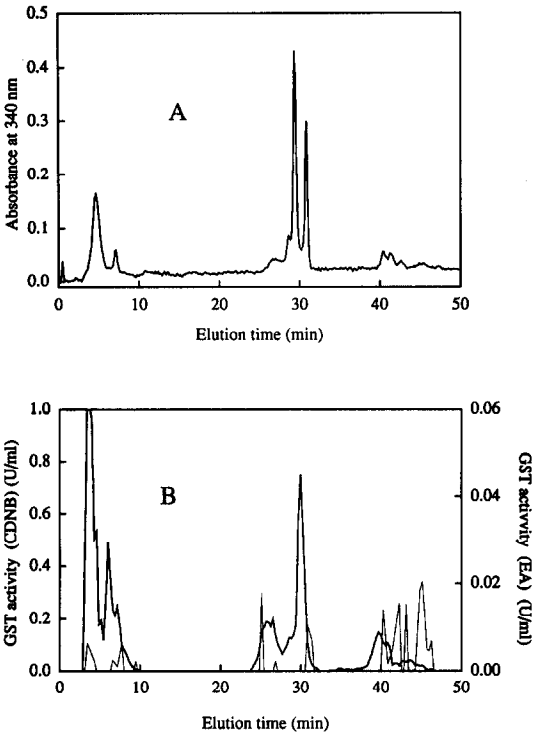

Fig. 3. Comparison of GST activity profiles obtained automatically and manually. The sample was a soluble fraction from fish (*Mugil* sp.) liver that had been purified by hexylglutathione affinity chromatography. The preparation had 66 U/ml GST. (A) 80 μl of a five-fold diluted solution were injected and the activity was determined automatically. (B) 100 μl of undiluted preparation were injected and the activity was determined manually in 300-μl fractions; the elution time was obtained by extrapolation from the elution volume. Heavy line: activity with CDNB; light line: activity with ethacrynic acid. Other conditions as in Fig. 2.

obtained in this way basically coincided with that obtained automatically although, as expected, resolution of the peaks was lower (Fig. 3). When CDNB was replaced by ethacrynic acid in the manual assay of fractions, most of the activity was found in group III and particularly around 47 min (Fig. 3B). A similar result was obtained by Lee *et al.* [17].

Comments

It has been argued [12] that the determination of GST isoenzymes by methods based on activity measurements is not as reliable as that based on protein subunit determination. The method described herein, with the automatic on-line detection of activity, can be performed in less than 3 h from the

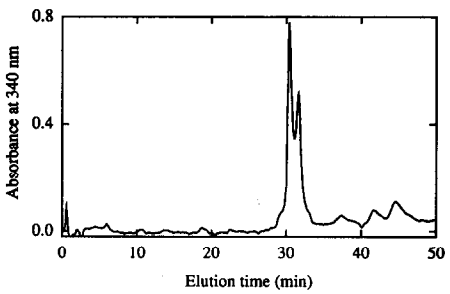

Fig. 4. GST activity profile of the fraction not bound to hexylglutathione–Agarose. A 100-μl volume containing 630 mU of GST activity were injected into the column. The sample was the fraction of cytosolic extract from fish liver that passed unretained through a hexylglutathione–agarose column. Other conditions as in Fig. 2.

intact tissue to production of the chromatogram (centrifugation at 30 000 *g* during preparation of the soluble fraction can be bypassed). This rapid processing allows for the control of activity losses. It gives information on isoenzymes as they are *in vivo* and not of the subunit components artificially separated by the denaturing conditions of sodium dodecyl sulphate–polyacrylamide gel electrophoresis or reversed-phase HPLC. Moreover, when an affinity chromatography step is included before the determination of isoenzyme content, some of the isoenzymes may be lost in the fraction which is not bound to the affinity matrix [6,9,12]. Actually, in this work, unbound GST typically accounted for 10% of the total activity recovered after the affinity chromatography under conditions such that the capacity of the column was not exceeded. Fig. 4 shows the activity profile obtained when the unbound fraction was run on our HPLC–DEAE system. The chromatogram showed two main peaks at 30.4 and 31.6 min with small shoulders at 29 and 33 min and minor peaks at 37, 42 and 45 min, thus supporting the idea that the affinity chromatography step discriminates against some isoenzymes of group II and group III present in crude extracts.

Van Ommen *et al.* [21] have pointed out that GST determination using CDNB does not properly express sample variability due to the lack of specificity of this substrate, whereas other substrates give differences of several orders of magnitude between certain isoenzymes. We believe that it is most convenient to use a universal substrate that gives the maximum activity with all the isoforms, provided

that each isoenzyme has been characterized *ad hoc*. To set a "specificity calibration" is the aim of present investigations.

In preliminary experiments, the method has worked satisfactorily for the detection of differences in the levels of individual isoenzymes in hepatic extracts from fish captured in contaminated and non-contaminated waters [22].

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