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Review

Methods for purification of glutathione peroxidase and related enzymes

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Abstract

The different preparative techniques and related analytical methods used for purification of glutathione peroxidase, glutathione transferase and glutathione reductase, described in papers published in the last ten years, have been reviewed in this article. Among the different purification techniques, chromatography has played a relevant role, being reported in all the papers reviewed, whereas other preparative techniques such as electrophoresis and isoelectric focusing were less employed and have been reported in only ca. 3% of cases. Frequently, several different chromatographic modes and several rechromatography steps have been employed. The use of at least three different chromatographic modes has been reported in 53% of total reviewed papers, whereas 41% of them employed two differents modes and in only 6% a single preparative chromatographic step was used. To evaluate losses and improve recovery, analytical methods for quantitation of protein and assay of enzymatic activity must be used in each purification step. Among these analytical techniques, gel electrophoresis, under denaturing conditions, has been widely used to assess purity of enzyme preparation. A discussion of the different activity assay methods used for these three enzymes is also presented in this article.

Keywords: Reviews; Glutathione transferase; Glutathione reductase; Glutathione peroxidase; Enzymes

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1. Introduction

Glutathione (γ-glutamylcysteinylglycine; GSH), discovered in 1921 by Hopkins [1], is now widely recognized for its important role in protecting cells from free radicals, peroxide, and xenobiotic-mediated injury. This important thiol is synthesized by the consecutive action of two enzymes, γ-glutamylcysteine and glutathione synthetases [2]. It has been known that GSH is present in almost all organisms and serves as a reductant in numerous biochemical reactions, including counteraction of oxidative events and protection of the thiol groups of intracellular proteins [3,4]

The active oxygen species which are produced in the cells contribute to the pathogenesis of several diseases [5]. Both prokaryotes and eukaryotes have defensive mechanisms against the toxicity shown by active oxygen species [6]. One important cellular antioxidant enzyme is glutathione peroxidase (GSHPx), first reported in 1957 by Mills [7], which catalyzes the reduction of hydroperoxides to their metabolizable hydroxyl forms with concomitant formation of oxidized glutathione (GSSG). GSHPx plays an important role in the prevention of the deleterious effects of peroxides, harmful intracellular metabolites, generated in the course of tissue metabolism [8]. Based on their substrate specificity, two types of GSHPx are generally recognized: selenium-dependent [9] and selenium-independent [10] enzymes. The Se-independent GSHPx activity is attributed to glutathione transferase (GST) isoenzymes acting on a variety of organic hydroperoxides [11], unlike the Se-dependent enzyme which is active with both organic and inorganic peroxides [12]. Both types are strictly specific for GSH as electron donor [13,14].

Reduction by NADPH of the GSSG produced by GSHPx during detoxification of hydroperoxides is catalyzed by glutathione reductase (GSSGRase), a widely distributed flavoprotein [15]. The reaction catalyzed by GSSGRase is very similar in chemical terms to those catalyzed by lipoamide dehydrogenase, trypanothione reductase and thioredoxin reductase, three closely related flavoprotein reductases which also catalyze the electron transfer between reduced pyridine nucleotides and low or high molecular mass disulfide substrates [16]. In addition to recycling the GSSG produced by GSHPx, GSSGRase has a central role in the intracellular GSH redox status, maintaining very high intracellular GSH/GSSG concentration ratios, according to the pivotal role of GSH for the optimum situation of the –SH/–SS– intracellular groups [16].

GSTs are a multigene family of ubiquitous dimeric enzymes which detoxify noxious organic compounds by conjugating GSH molecules [17–19], and are implicated also in several endogenous functions [20,21]. In terms of structure, function and tissue distribution [22–25], rat GSTs provide the standard for comparison with GSTs from other organisms and have been grouped into four classes, α, μ, π and θ, according to their physiological, structural and genetic similarities [26,27]. In addition to using GSH as a common substrate, some GST isoenzymes also display Se-independent GSHPx activity [28]. This dual activity is explained by the close proximity existing in GSTs between the highly conserved GSH-binding site and the less specific hydrophobic substrate binding area [29]. This allows the nucleophilic attack of GSH leading to adduct formation, characteristic of GST activity, and the reduction of organic hydroperoxides, typical of GSHPx activity.

In this article, the different preparative techniques and related analytical methods, utilized for the purification of these three GSH-requiring enzymes have been reviewed, mainly those described in papers published in the last ten years. A simplified scheme of the reactions catalyzed by these enzymes is shown in Fig. 1.

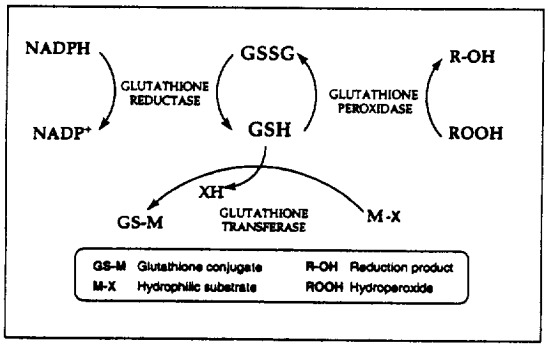

Fig. 1. Reactions catalyzed by GSHPx, GST and GSSGRase.

2. The purification process: a brief overview

The protocols utilized for the purification of these three enzymes, rely on separation techniques that advantageously exploit the differences in chemical structure and surface properties of proteins. The main objective is to isolate the enzymes from a complex mixture with a very high recovery of mass and activity. A battery of analytical and preparative separation techniques is required to purify a protein to homogeneity.

In preliminary steps, the enzymes must be extracted from their biological sources and a wide number of extraction procedures can be utilized for this purpose. The samples derived from tissues, bacteria, physiological fluids etc., require the removal of particulate contaminants prior to the performance of any other separation step. Creative use of these extraction and sample handling techniques, such as ultracentrifugation, fractional precipitation, selective removal of proteases etc., may frequently improve the overall purification protocol.

Among the different separation techniques used with these enzymes, chromatography plays a relevant role. The use of this technique has been reported in all papers reviewed, whereas other preparative techniques such as electrophoresis and isoelectric focusing (IEF) were reported in only 3% of cases. The different chromatographic modes employed for purification of these enzymes fall into five groups: affinity, ion-exchange, hydrophobic and reversed-phase, hydroxyapatite and size-exclusion; these five chromatography types and the most frequently used supports are schematically shown in Fig. 2. Frequently, several different chromatographic modes and several rechromatography steps are used. At least three different chromatographic modes have been used in 53% of the reviewed papers, whereas 41% employed two differents modes and the use of a single preparative chromatographic step was reported in only 6%.

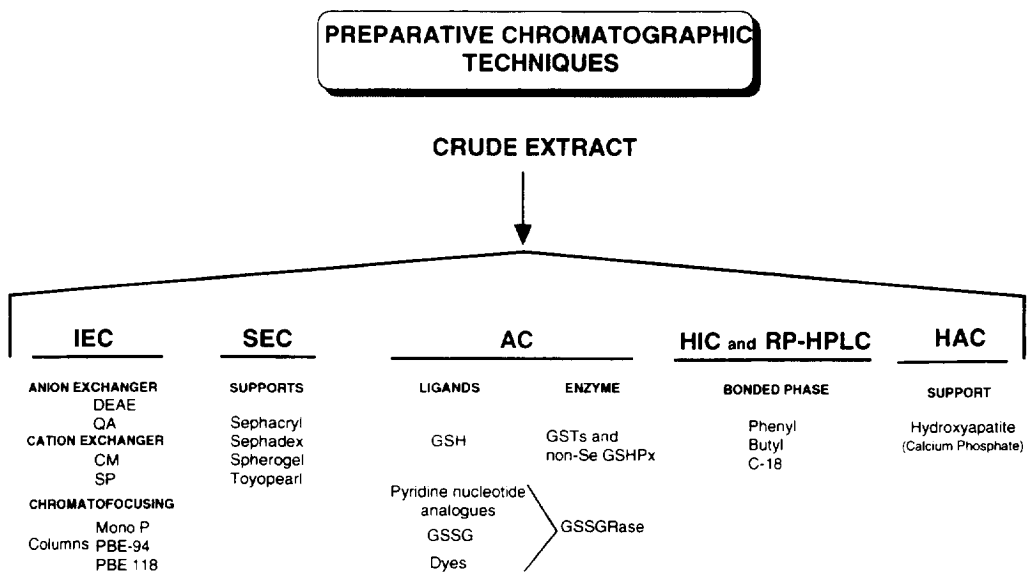

Fig. 2. Chromatographic modes and supports used in purification protocols for GSHPx, GST and GSSGRase.

Solvent exchange is necessary after each chromatographic process before the next chromatographic step. A sample recovered from hydrophobic interaction chromatography (HIC) will not usually adsorb to an ion-exchange chromatography (IEC) column, since the high salt concentration in the sample will not allow such interaction. Dialysis or solvent change using size-exclusion chromatography (SEC) on Sephadex G-25 columns is, thus, required for samples eluted from IEC, HIC and AC. Ultrafiltration using 10 000 relative molecular mass cut-off membranes is useful to concentrate the enzyme pool prior to the next chromatographic step.

To evaluate losses and improve recovery, analytical methods for quantitation of protein and assay of enzymatic activity must be used in each purification step. Protein assay methods, such as Bradford [30], Lowry et al. [31] or bicinchoninic acid [32], have been widely used for this purpose. A discussion of the different assay methods used for these three enzymes is presented in Section 4.2 of this article.

3. Preparative techniques

3.1. Extraction procedures

Cell disruption is usually the first step for obtaining intracellular enzymes. Several methods, including homogenization, sonication, French press, grinding, vortexing with glass beads, or lysis with detergents, organic solvents or osmotic shocks [33–38], are useful for this purpose. The complexity of biological samples accounts for the need for some pre-treatments in order to achieve good separations. Fractional precipitation with ammonium sulfate has been successfully used, and the enzymes reviewed can be isolated in the 30–70% saturation fraction. Ultracentrifugation (at least 100 000 *g*) is an important step to obtain clear cell-free extracts. Beneficial steps for the extraction process of GSHPx are acid treatment and heat precipitation, which precipitate a large amount of non-glutathione peroxidase proteins [39]. Acetone precipitation partially purifies and concentrates the sample for application onto chromatographic columns. Dialysis or SEC are used for buffer exchange. Actually, several of these pretreatments are required before chromatography or electrophoresis is carried out.

To preserve enzymatic activity, each purification step should be optimized in relation to the type of buffer, pH, ionic strength, temperature and additives. Buffer systems based on Tris–HCl (pH 7.5–8.5) or phosphate (pH 7.0–7.5) containing EDTA and other additives have been the most used. To prevent microbial contamination, sodium azide is often added to the buffer at 0.2–0.3%, concentrations which do not affect most proteins. Many enzymes lose enzymatic activity upon oxidation, which is usually restored by adding thiols, such as 2-mercaptoethanol (5–15 m*M*) or dithiothreitol (DTT) (1–5 m*M*), to buffer. Thus, GSHPx isolated from *Chlamyomonas reinhardtii* [40] was so unstable that it was fully inactivated after three days; addition of 5 m*M* GSH to buffer allowed the enzyme to retain 85% of the original activity after twenty days. Addition of an inert protein, such as bovine serum albumin (BSA), is usually required to prevent losses due to unspecific adsorption to glass surfaces. Since intracellular proteases are released by cell disruption, protease inhibitors such as phenylmethanesulfonyl fluoride (PMSF) must be added to the buffers to avoid protein degradation.

An appropriate choice of cell disruption, homogenization, fractional precipitation and ultracentrifugation methods can yield purification factors of the order of two- to six-fold for the enzymes reported in this article. Although these initial methods have inherently poor discrimination power, the most important benefit is the removal of other classes of biomolecules such as lipids, polysacharides and nucleic acid components.

3.2. Chromatographic procedures

Preparative-scale liquid chromatography has rapidly became the method of choice for purification of biological macromolecules. The highly efficient column technology, previously found only in analytical HPLC, can now be used on a preparative scale. Whereas in analytical applications the emphasis is placed on resolution and duration of analysis, in preparative chromatography the amount of enzyme isolated and its purity are critical parameters. The main goal of any preparative process is to obtain the largest quantity of enzyme, with the highest purity, in the shortest time. The chromatographic separation

of a complex mixture of proteins can only be achieved by using high-efficiency columns.

Previous chromatographic schemes contained long and complicated steps, required large homogenate volumes and resulted in low final yields. For instance, conventional IEC on DEAE cellulose requires up to 38 h, while size-exclusion chromatography and chromatofocusing (CF) each require about 13–18 h. New rapid procedures have been recently developed, yielding high resolution and resulting in complete recovery of enzymatic activity. A plethora of books, reviews and articles dealing with the applications of chromatographic methods in protein chemistry have been published but are outside the scope of the present review.

The first paper using HPLC for resolution of complex protein mixtures appeared in 1976 [41], but a widespread use of this technique was severely limited by the absence of proper stationary phases, and a considerable amount of effort was made to improve them. The soft supports used for conventional liquid chromatography, such as cellulose, agarose, or dextran, have severe drawbacks and are not suitable for HPLC. The new HPLC media minimize nonspecific interactions and withstand the high pressures and flow-rates of HPLC, while operating at room temperature. Almost all chromatographic methods used for protein separation on soft gels have been adapted for HPLC separations. Nowadays, modern HPLC systems can be used to conduct rapid separation of complex protein mixtures.

Silica [42,43] and polymer-based supports [44,45] are regularly used for protein separation by HPLC. These packings can be prepared in particle sizes from 5 to 50 μm and pore diameters from 70 to beyond 1000 Å [46], and are stable in the pH range 2–7.5 for silica and 2–12 for polymeric supports. The supports can be derivatized and prepared for most chromatographic modes [47,48]. Separations of complex protein mixtures by porous packings are ten-fold faster than by soft gels with very high enzyme recovery (greater than 85%) in most cases. In addition to speed and resolution, non-porous packings have several features which make them powerful tools for fast protein separations. Their mass recovery is equal or superior to that of porous columns with significantly reduced analysis times. The small column volume also reduces the amount of mobile phase needed by as much as 80%. Due to the high cost of such columns, the use of guard-columns is highly recommended.

Corrosion in wetted parts of the equipment and low protein recoveries were frequently reported in early papers describing the use of HPLC for protein purification. During the last decade, manufacturers have paid particular attention to designing HPLC equipment with wettable parts made with inert materials (Teflon, Kel-F®, polyethylene, polyetheretherketone (PEEK), etc.) instead of stainless steel and, in general, suitable for use in a cold room. They are named "biocompatible systems" and are available from several HPLC manufacturers: Pharmacia, Perkin-Elmer, Waters and Beckman Instruments. A wide range of automated HPLC equipment is now available for continuously monitoring the eluate from the chromatographic column and for carrying out protein purification from complex mixtures, within hours instead of days, as was usual for conventional liquid chromatography.

With the introduction of "biocompatible" instruments into the market, scientists are faced with the decision of whether to get such an apparatus or to continue with the classical stainless-steel HPLC instruments. It is true that metallic cations do inhibit the activity of a certain enzyme, but if an enzyme is sensitive to such interferences, the addition of chelating agents will easily solve the problem. In relation to the adsorption of enzymes on stainless-steel surfaces, it should be noted that the proteins adhere to any surface, stainless steel, glass, titanium, teflon, plastic, etc. In our opinion, biocompatibility is not a substantial problem in protein chromatography and the performances of stainless-steel systems are comparable to those of inert systems. If no corrosion problems exist and no significant adsorption or inactivation of proteins is detected, the use of classical HPLC equipment should not be excluded. In our laboratory, we have successfully used classical HPLC equipment for the purification of several enzymes by affinity [49] and immunoaffinity chromatography [50] with excellent results.

3.2.1. Affinity chromatography

In affinity chromatography (AC), proteins are isolated on the basis of their biospecific interaction with a complementary immobilized ligand. Among the chromatographic techniques, AC offers the great-

est specificity and selectivity for enzyme isolation, being usually carried out at final purification stage, mainly due to the cost and lability of affinity media. Enzymes can be recovered with high yields of activity provided that very clear samples are used. The principles and applications of AC have been reviewed in detail [51–54].

New supports for AC contain activated groups such as epoxy, hydroxyl, tresyl, nitrophenyl and N-hydroxysuccinimide, which form stable covalent linkages with a variety of affinity ligands through appropriate functional groups, such as thiol, hydroxyl and primary or secondary amines [51–54]. Access of the coupled ligand to the enzyme binding site is improved by intercalating spacer arms (1,6-diaminohexane or 1,4-diaminobutane) between the ligand and the support backbone [51,53]. Elution from the affinity column can be accomplished either by unspecific or specific means, using gradient, pulse or isocratic modes. Changes in temperature, ionic strength, pH, and concentration of chaotropic ions or organic solvents in the mobile phase are suitable for unspecific desorption of enzymes. For specific elution, an appropriate counter ligand is added to the mobile phase.

Modern high-performance liquid affinity chromatography (HPLAC) combines the biospecificity of conventional AC with the speed of operation, resolution and sensitive detection of HPLC techniques [43]. The HPLAC methodology allows multiple runs in a day with very good reproducibility, high recovery, and the process can be carried out in most cases at room temperature. We have used a stainless-steel prepacked epoxy-activated silica Ultraffinity® column from Beckman, for one-step partial purification of GSSGRase from different cell-free extracts, with high recoveries and excellent purification factors [49].

GSH can be bound through either its thiol or amino groups to a variety of activated supports [55–57]. GSH-based affinity supports have been successfully used for the isolation of GSH-requiring enzymes. Thus, immobilized GSH has been used for the purification of GSTs [58–65] and non-Se-dependent GSHPx [66–68], whereas immobilized GSSG has proved to be useful for GSSGRase [55,56]. With this last affinity support, GSSGRase bound to the column, must be eluted using NADPH rather than by GSSG. It should be noticed, however, that the use of NADPH in the elution buffer will convert the immobilized GSSG to GSH, thus altering the specificity of the column for further chromatography. High concentrations of $NADP^+$ in loading buffer can also elute the enzyme. This affinity system exhibits an unusual biospecific behaviour in which the enzyme is bound to an immobilized substrate but released by its soluble coenzyme [56].

GSH affinity media have been reported as highly useful to isolate GSTs from a wide variety of sources, previous to further studies, such as assay of specific activities and isoenzyme composition using reversed-phase HPLC (RP-HPLC). In most cases elution can be accomplished using up to 5 m*M* of counterligand, either GSH or S-hexyl-GSH [60,63,67,69,70]. Typical dynamic capacity for these supports is ca. 0.2 mg of enzyme per ml of bed gel [61]. Bromosulfophthalein–GSH agarose affinity chromatography is an useful alternative for the separation of anionic and cationic GST isoenzyme forms from human erythrocyte, heart and lung as reported by Singh et al. [64,71]. The enzyme, unabsorbed to this support, represents the anionic isoenzymes. In our laboratory, GSH affinity chromatography is a routine technique for the isolation of fish GSTs. An elution pattern from an S-hexyl-GSH affinity column is shown in Fig. 3. A single step procedure using affinity chromatography on S-hexyl GSH has been used to purify GST from *E. coli* mutants whose cysteine residues were independently substituted with alanine by site-directed mutagenesis [72] and for the purification of class 8–8 GST dimers from different rat tissues [73]. In this method, the bound isoenzymes were selectively eluted from the column using 5 m*M* of GSSG. This chromatographic technique is highly selective and can be used as the initial chromatographic step with clean samples such as crude extracts after ultracentrifugation or, in most cases, it can serve as a final purification step.

As with other pyridine nucleotide-dependent enzymes, GSSGRase can be purified using those affinity supports which are useful for $NADP^+$-related enzymes. Specific group ligands, such as dyes [51–54,74] and pyridine nucleotide analogues [49,75–79], have been widely used for this purpose. Since orthophosphate is a competitive inhibitor of several $NADP^+$-dependent enzymes, Phospho-Cellu-

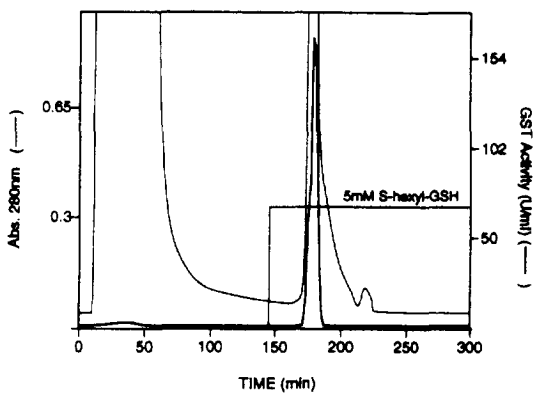

Fig. 3. Affinity chromatography of GST on S-hexyl-GSH agarose fast-flow. Cytosol of grey mullet liver (22 ml, 808 Units) was applied to the affinity column (6.4 × 1.0 cm I.D.) equilibrated with 50 m*M* potassium phosphate buffer, pH 7, containing 1 m*M* EDTA and 2 m*M* DTT. The column was washed with the same buffer containing 50 m*M* KCl and eluted with 50 m*M* Tris–HCl buffer, pH 9.6, containing 5 m*M* S-hexyl-GSH. Activity was measured with CDNB; flow-rate, 1 ml/min; protein detection, 280 nm; equipment, Waters 650E Advanced Protein Purification System.

lose is also useful for enzymes with affinity towards phosphorylated substrates [80,81]. Takagahara et al., have reported the use of this affinity media for the purification of yeast GSSGRase [81]. In some cases, affinity chromatography has been used as a unique chromatographic mode for purification of this enzyme. Thus, López-Barea et al. used two sequential AC steps with pyridine nucleotide analogues [adenosine-2′,5′-diphosphate (2′,5′-ADP) and 8-[6-aminohexyl]-amino-2′-phosphoadenosine-5′-diphosphoribose (C8ATPR)] for purification of GSSGRase, with 66% yield and 4695-fold purification for the mouse liver enzyme [75] and 63% recovery and 2632-fold purification for the *E. coli* enzyme [78].

3.2.2. Ion-exchange chromatography

Separation in IEC is achieved on the basis of either charge or isoelectric point of proteins and IEC is frequently used as an early purification step because of the high loading capacity of supports and its ability to remove many contaminants of different electric charge. Commercial polymeric IEC supports are available in bulk or in prepacked columns, can operate in a pH range 2–13, have particle sizes 8–40 μm and pore diameters to beyond 1000 Å. They show typical ion-exchange capacity of 100 to 250 μeq/ml and protein binding capacity of ca. 60 mg/ml. These packings reduce non-specific adsorption, resulting in quantitative recovery of protein mass and, for cleaning purposes, withstand exposure to 0.5 *M* sodium hydroxide or 10–30% acetic acid solutions. Commercial IEC columns for proteins offer excellent resolution, quantitative recovery of biological activity and short separation times. They have a low resistance to flow and hence can operate at moderate pressures.

Anion-exchange supports are made with either diethylaminoethyl (DEAE), polyethyleneimine (PEI) or quaternary ammonium (QA) groups, and cation exchangers contain either carboxymethyl (CM) or sulfopropyl (SP) groups. Based on their pH-dependent ionization, DEAE, PEI and CM exchangers are considered "weak" and QA and SP "strong". Strong ion exchangers are ionized over a wide pH range and exhibit slightly better selectivity than weak exchangers. Protein elution from IEC columns is usually carried out by salt gradients (frequently up to 0.5 *M*). In some cases, the polymeric support matrix exhibits a certain hydrophobicity, and unspecific retention of proteins may occur. Such unwanted interactions can be avoided by using a convenient concentration of chaotropic salts in the loading buffer [82].

In efficient two-step protocols for the purification of GSTs, AC is usually followed by anion-exchange HPLC [64,83], which provides a rapid separation, which is necessary to minimize and/or to eliminate possible formation of oxidation or degradation products that can be erroneously interpreted as new GST isoenzymes. Thus, Radulovic and Kulkarni [65] have reported a rapid method for purification of human placental GST using AC on hexyl-GSH and a HPLC anion exchange on a Synchrom AX 300 column, with excellent activity recovery (124%) and 1342-fold purification. Samples eluted from the affinity column, containing up to 1 mg (about 112 units of enzyme activity), could be processed in each chromatography with this column. Singh et al. [64] have reported the purification of human erythrocyte, heart and lung GST isoenzymes using a similar protocol; the column used in this case was a SynchroPak AX-300 which recovered 100% of the enzyme activity.

Protein separations based on their isoelectric points is successfully carried out by CF [84–87]. In this chromatographic mode an artificial pH gradient is created within the column and this is used for protein elution instead of the increased ionic strength exploited in conventional IEC. The CF technique is carried out on special supports, bearing immobilized anion-exchange groups of broad titration curves which provide inherent buffering capacity to the matrix. In addition to purification, CF is highly specific and has high resolution for separation of isoenzymes and is suitable for determination of their isoelectric points. Among the different supports suitable for this methodology, PBE and Mono P type columns from Pharmacia have been widely used to resolve the different isoforms of GSH-requiring enzymes [21,60,64,74,76,88,89]. Thus, a procedure described by Vander Jagt et al. [89] to isolate thirteen forms of GSTs from human liver, reported the use of AC on GSH coupled to epoxy-activated Sepharose and two CF steps, the first using a PBE 118 column to focus protein in the range pH 10–7.5 and the second on a PBE-94 column to focus in the pH range 7.5–4.5. The isoenzymes were purified 15 000-fold with a 77% recovery. This technique, using fast protein liquid chromatography (FPLC) on a Mono P column as a final chromatographic step, has been also reported by Ålin et al. [25] for the purification of cytosolic GST isoenzymes from rat liver.

3.2.3. Hydrophobic and reversed-phase chromatography

Hydrophobic interaction chromatography, introduced by Hjerten [90], is a versatile technique for the separation of proteins based on differences in their hydrophobicity. HIC and RP-HPLC are similar since, in both modes, solute retention derives from hydrophobic interactions with the packings, while they differ in the strength of interaction between proteins and supports. RP-HPLC uses packings with highly dense and lipophilic bonded phases, producing a very strong protein interaction. Consequently, organic solvents are required to elute the proteins, resulting in denaturation and loss of biological activity, since renaturation is a very slow process which is not achieved in many cases [91]. The lower ligand density and hydrophobicity of the bonded phases for HIC result in mild interactions with proteins, and elution is accomplished with saline buffers instead of organics. Thus, in HIC, most proteins undergo little (if any) denaturation and are recovered with their biological activity intact.

Proteins are only retained by the HIC support at high salt concentrations which favour hydrophobic binding, and elution is carried out by decreasing salt gradients in the mobile phase. An excessive hydrophobicity produces severe retention, leading to band broadening, mass loss or protein inactivation. In decreasing order of hydrophobicity, phenyl, butyl, propyl and hydroxypropyl are useful bonded phases for HIC of proteins. Selectivity can be increased by controlling the type and concentration of salts, and the pH, temperature and gradient shape. The mild adsorption and elution conditions of HIC result in high protein recovery and provide one of the most suitable techniques for enzyme purification.

RP-HPLC is an excellent tool to assess purity and to prepare enzymes for sequencing. In spite of its limitations, RP-HPLC has been used as a final step to obtain pure enzyme. Esworty et al. [92] have used this technique to obtain pure GSHPx. The column used was a Brownlee RP 300 (30 cm × 4.6 mm I.D.), recovering between 6 and 8 μg of pure enzyme in each chromatographic process. The peak eluted from this column was identified by means of amino acid analysis.

The separation of different GST subunits is usually carried out by RP-HPLC. Ostlung Farrants et al. [63] reported a method using C_{18} columns, which separates at least two different families from rat tissues, μ and π. The procedure is rapid and sufficiently sensitive to measure 5 μg of each subunit in a mixture. Improved resolution and increased yield (ca. 100%) has been reported by Meyer et al. [73] using columns with 300 Å pore size. We have successfully used this technique as routine in our laboratory to analyze the subunit composition of GST from fish liver. Fig. 4 shows the elution profile of GST subunits from grey mullet liver.

3.2.4. Size-exclusion chromatography

In SEC, proteins are separated on the basis of their apparent size and shape in solution. SEC is a non-interactive technique, has a wide applicability in protein purification and is used to fractionate the

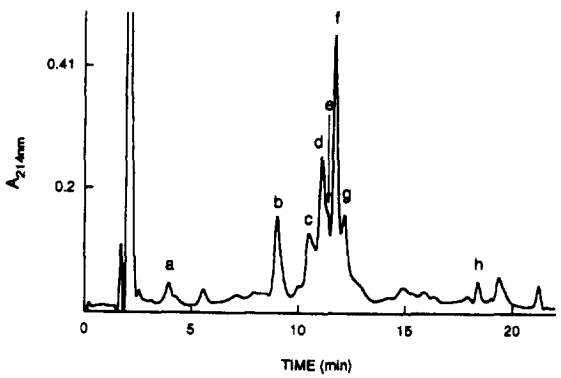

Fig. 4. Subunit composition of GST from grey mullet liver. Column, Vydac C4 214 TP (15 × 0.46 cm I.D.); sample, 20 μl of eluate from S-hexyl-GSH agarose affinity column; Flow-rate, 1 ml/min; elution, linear gradient 40–70% acetonitrile–water containing 0.1% trifluoroacetic acid lasting 30 min; equipment, Beckman System Gold Model 126 chromatograph with a 168 diode array detector.

components of a sample with minimal loss or degradation of proteins. The molecular sizes of all components of the protein sample must fall within the fractionation range of the support. The SEC packings available for HPLC have particle sizes of 5–10 μm with pore sizes up to 10^4 Å, are stable over the pH range 2–13 and resist up to 6000 p.s.i. (~1 Pa). Sample load is low to moderate, the capacity of many packings reaching up to 1 mg protein/g of support. HPLC–SEC columns are often four- to five-fold bigger than standard (25 cm × 4.6 mm I.D.) reversed-phase columns, and 10- to 20-fold bigger than IEC columns. It can also be used for desalting or changing the sample buffer, as salts are easily separated from the much larger proteins. An arrangement of two HPLC–SEC column in series has proved to be highly efficient for the determination of protein size in solution and, in some instances, it has been reported as a crucial purification step [93,94]. Thus, Lyons et al. [95] have reported a rapid, high-yield purification of rat liver GSH peroxidase using HPLC, in which the crucial step was SEC on a TSK 3000 SW (30 cm × 7 mm I.D.) column, the enzyme being eluted as single peak. A 3500-fold purification and a 42% recovery of the initial enzyme activity was reported by the authors.

3.2.5. Hydroxyapatite chromatography

Hydroxyapatite chromatography (HAC) has been shown to be helpful for the purification of proteins, specially when other chromatographic modes were not successful. Hydroxyapatite is a crystallized form of calcium phosphate and has properties similar to a mixed mode ion-exchange support. This support binds acidic, basic and neutral proteins. The elution conditions for these columns are not essentially different from those for IEC and hence, saline linear gradients are frequently used. Although both techniques are similar, the separation mechanisms are not the same and the order of elution of peaks is different in most cases. The major advantage of HAC is its high capacity and versatility. Main drawbacks to this technique are very short column lifetimes, due to the fragile nature of the support and pressure limitations, hence HAC cannot be used at flow-rates higher than 1 ml/min. In some cases, a strong adsorption of proteins is observed. Despite these limitations, this chromatographic mode is widely used nowadays as an important step in the purification of GSHPx and GST enzymes [40,89,94,96,97], although it is infrequently employed for GSSGRase. New HPLC-hydroxyapatite supports such as Bio-Rad's ceramic Macro-Prep® overcome the instability and pressure limits of the traditional form of this material. Nevertheless, no applications of this media to the enzymes selected in this review have yet been reported.

3.3. Other preparative techniques

Preparative electrophoresis has not been used frequently in the purification protocols reviewed, since separations by electric charge or p*I* can be also successfully obtained using ion-exchange chromatography or CF; these techniques are less complicated, faster and able to isolate larger quantities of protein than electrophoresis. Nevertheless, when a pure enzyme preparation is required for antibody production or protein sequencing, different modes of preparative electrophoresis, IEF [98] or isotachophoresis [99] achieve highly efficient purifications that could not be easily performed in a single step by any other separation technique. Since separation depends simultaneously on both size and charge, proteins that are not separable under the usual SEC,

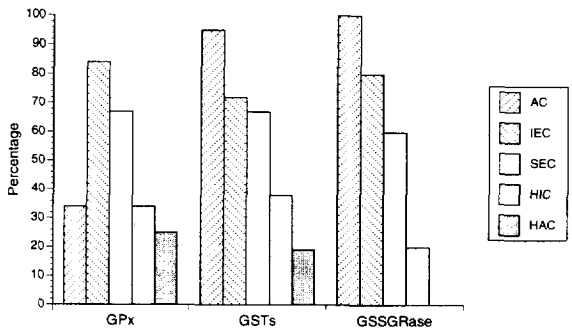

Fig. 5. Percentage of utilization of different chromatographic modes for each of the enzymes described in the present review.

IEC or HIC methods can be resolved by this system [100,101]. Preparative isoelectric focusing can be carried out in horizontal electrophoresis systems using Sephadex gel plates [102] or in gel columns containing the selected ampholites in a sucrose (up to 50%) gradient. In this last case, focusing is achieved using electric fields up to 1500 volts during 17 to 28 h [64,10,104]. Preparative IEF is a powerful technique used as a final purification step. Di Ilio et al. [58] have reported the use of this technique to obtain pure GST from *Xantomonas campestris* to subsequently study the subunit composition using RP-HPLC, sequence the N-terminal and estimate the relative fraction of secondary structure using circular dichroism.

As previously stated, chromatography has a prominent role as a preparative technique in the protocols for the purification of these GSH-requiring enzymes. Fig. 5 shows the percentage of utilization of different chromatographic modes for each of the enzymes described in the present review. More detailed information about the use of different chromatographic media, within each chromatography mode, for these enzymes is shown in Fig. 6.

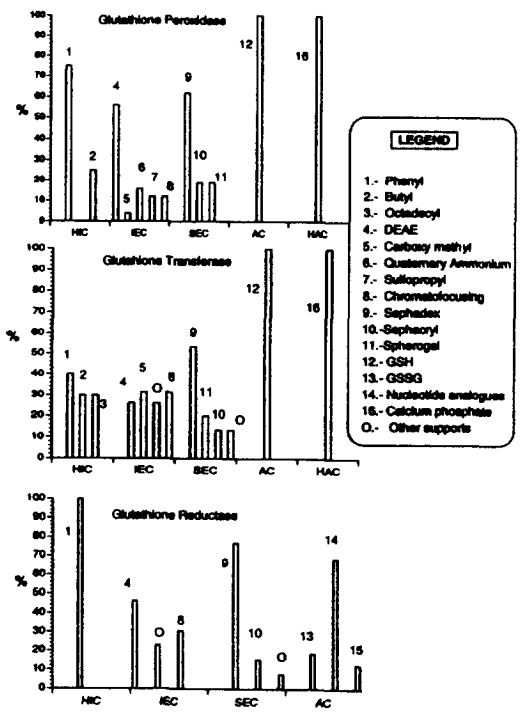

Fig. 6. Use of different chromatographic media. 1 = phenyl; 2 = butyl; 3 = octadecyl; 4 = DEAE; 5 = carboxy methyl; 6 = quaternary ammonium; 7 = sulfopropyl; 8 = chromatofocusing; 9 = Sephadex; 10 = Sephacryl; 11 = Spherogel; 12 = GSH; 13 = GSSG; 14 = nucleotide analogues; 16 = calcium phosphate; O = other supports.

4. Analytical methods

4.1. Electrophoretic methods

The differential mobility of proteins in an electric field is the basis of electrophoretic separations which are widely used in biochemistry to determine purity, molecular mass and p*I* of proteins [105–107]. Modern protein electrophoresis began with the experiments of Tiselius on moving boundary electrophoresis [108,109], requiring tens of milligrams of sample for detection. Over the last decades, much work has been done to develop equipment to carry out different electrophoretic modes, such as zonal [110], isotachophoresis [111], isoelectric focusing [112,113] and capillary electrophoresis (CE) [114,115]. Most electrophoretic methods were initially conceived for analytical purposes [116], and were not widely used on a preparative scale [98,117,118]. Polyacrylamide is the polymer most used for gel electrophoresis [119]. Different polyacrylamide gel electrophoresis (PAGE) modes such as homogeneous, discontinuous or gradient systems, under either native or denaturing conditions or even two-dimensional systems, were designed to quantitate, compare and characterize enzymes [120–126]. Today, gel electrophoresis is a well-established

routine technique in all protein laboratories, and equipment for different electrophoretic modes is commercially available from several manufacturers, such as Bio-Rad, Hoefer, CBS Scientific Company, Applied Biosystems, Novel Experimental Technology, Pharmacia etc. Usually quantitation is carried out by densitometry. Among the different electrophoretic techniques, sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE) is the most frequently used method to check the homogeneity of any enzyme preparation and has been reported in all the papers reviewed.

One of the major limitations of gel electrophoresis is the Joule heating effect due to the electric field applied. The poor heat dissipation of gels only allows their use at relatively low potentials and hence is considered as a slow separation technique. In most cases, the use of anticonvective gels and cooling devices considerably improves protein resolution [127].

Modern CE [128–132], essentially free of the limitations observed in conventional gel electrophoresis, allows on-line detection of analytes and therefore real-time data analysis and automation. The state of the art of CE technology has been reviewed in detail [133–137]. High-efficiency separation of proteins can be now carried out using buffer additives and coated capillaries. Coated capillaries that diminish protein–wall interactions to separate proteins at the femtogram levels are available from Applied Biosystems, BioRad, Supelco Inc. etc., and gel-filled capillaries from Beckman Instruments, Applied Biosystems and J&W Scientific. As far as we know, the use of CE in the analysis of the three reviewed enzymes is limited. In 1994 Guttman and Nolan [138] compared the separation of many proteins by SDS-PAGE in slab gels and SDS-PAGE gel-filled capillaries. The behaviour of GSSGRase was different in both systems: subunit M_r values were $62.2 \cdot 10^3$ and $54 \cdot 10^3$ for capillary and slab gel systems, respectively, both differing with those previously published ($52 \cdot 10^3$) for this enzyme [139].

After running the gels, the protein bands are localized by Coomassie [140–142] or silver stains [143,144]. Silver staining is about 100-fold more sensitive than Coomassie Blue, although the differential staining obtained for different proteins with silver is an important drawback [60,76,92,103]. The relationship between density of silver staining and protein concentration is characteristic for each protein and, thus, pure enzyme should be included in each gel to quantitate a particular protein. Coomassie staining yields the same color intensity for different proteins and is usually recommended for densitometric quantitation, while silver staining is useful to detect minor contaminants, Several methods have been developed to reduce the excessive background of gels and to improve protein quantitation [145].

Assignment of individual bands is usually carried out by in situ staining for specific enzyme activities. Several methods have been developed to detect activities in gels and a number of books and review articles have been published [18,146,147]. Electrophoresis in denaturing conditions yields better resolution than other gel electrophoretic modes, but enzyme activity is irreversibly lost in most cases. Thus, non-denaturing conditions are highly recommended in all steps of the electrophoretic process to localize enzyme activity bands. Renaturation of proteins to recover enzymatic activity after SDS-PAGE by removing the detergent, has been reported for monomeric and homodimeric enzymes [148]. Nevertheless, this method allows only a partial restoration of activity, although usually enough to perform a positive stain [149]. After SDS-PAGE, enzyme bands can be visualized using immunoblotting techniques [150–152]. A suitable method to detect GSHPx from rat hepatocytes have been described in 1994 by Asayama et al. [68]. In brief the bands on SDS-PAGE are electrophoretically transferred to a nitrocellulose membrane and incubated with anti-GSHPx antiserum. After this treatment, a new incubation at room temperature with goat anti-rabbit IgG conjugated to horseradish peroxidase is needed. Color development can be carried out using either the diaminobenzidine reaction [153], 4-chloro-1-naphthol and hydrogen peroxide [76], or alkaline phosphatase conjugated anti-rabbit IgG and *p*-nitrotetrazolium dye [150]. Immunoblotting has been also applied to detect GST bands after electrophoresis [69]; in this case, the enzyme–antibody complex can be revealed by autoradiography using ^{125}I-radiolabeled protein A. Electrophoresis under native conditions requires protecting the thiol residues, thus, either a pre-electrophoresis or polymerization with riboflavin and light instead of peroxides is recommended [154].

Autochromic methods have been also widely used to detect enzyme activity bands. The progress of the enzymatic reaction can be directly followed by observation of changes in light absorption or fluorescence of either substrate or product. Thus, GST activity can be visualized in the gel as yellow bands using 1-chloro-2,4-dinitrobenzene (CDNB) as acceptor substrate [155,156]. An useful method to assay GSPx in native PAGE gels have been reported by Zakowski and Tappel [97]. After electrophoresis, the gel is incubated with the substrates, GSH and cumene hydroperoxide, and revealed with a 50% saturated nitroprusside solution in ammonia. Non-stained areas are indicative of GSPx activity. In other methods, GSPx activity bands can be visualized under UV light using the coupled reaction with GSSGRase; activity bands may be detected by the quenching of the NADPH fluorescence [157,158]. Laser densitometers are useful for scanning the protein colored band in the gels [59,159].

GSSGRase activity bands can be detected by using the methods described for other NADPH-dependent enzymes. Visualization of GSSGRase bands, based on the reduction of tetrazolium dyes by NADPH in presence of 2,6-dichlorophenolindophenol (DCIP) to produce insoluble colored formazans, was reported in 1968 by Kaplan [160]. This method can detect diaphorase activity as well as GSSGRase. When thiols such as 2-mercaptoethanol were included in the gel buffer, the enzyme activity was detected under UV light by loss of fluorescence produced upon conversion of NADPH to $NADP^+$ [161].

4.2. Activity assay methods

Enzymatic activity assay methods can be classified as continuous or discontinuous. In a continuous method, direct observations are made within the assay mixture while the reaction is going on. In a discontinuous method, the reaction is followed by withdrawing samples at various times, and the assayed parameters are determined after stopping the reaction. Assay methods using separation techniques can be essentially considered as discontinuous.

4.2.1. Continuous assay methods

The substrates or products of many enzyme reactions may absorb light, either visible or ultraviolet. Since it is unlikely that they would have identical spectra, it is possible to find a wavelength to measure the progress of the enzymatic reaction at which the conversion of a substrate into a product is reflected by an absorbance change. Spectrophotometric assays are usually preferred to any other due to their facility and sensitivity, enough for most applications. Whenever either the products or the substrates cannot be continuously followed in the initial reaction mixture, a discontinuous assay method is necessary to monitor the reduction rate.

The most commonly used assay for GSHPx is the photometric coupled method described in 1967 by Paglia and Valentine [162], in which GSSG formed by the enzyme is reduced to GSH by GSSGRase and NADPH. The reaction rate can be followed spectrophotometrically by monitoring the decrease in absorbance at 340 nm. Several modifications of this assay, which vary in the type of peroxide, temperature, pH, and presence of inhibitors and chelating agents [163–165], have been widely used. Under the experimental conditions used for these assays, the enzyme cannot be simultaneously saturated with respect to both GSH and hydroperoxide, [166] and the observed activity depends on the substrate concentration utilized. Therefore, the different versions of the standard coupled assay, can yield different results. This lack of a standard enzyme unit definition has hampered the interlaboratory comparisons of results [167].

Other assay methods for this enzyme based on the direct measurement of GSH consumption [168,169] or indirect, via the reaction of the sulfhydryl group with the Ellman's reagent 5,5′-dithio-bis-[2-nitrobenzoic acid] (DTNB) [170] have been reported. None of them is useful for kinetic studies, since NADPH is an inhibitor of GSSGRase [16] and the DTNB reaction is very tedious [171]. A semiautomated assay using DTNB, to continuously monitor the GSH consumption has been reported by Zakowsky and Tappel [171] and was used to study the reaction kinetics. The method was applied for the assay of relatively purified enzyme samples, so that no protein precipitates when the sample is mixed with trichloroacetic acid. This method allows the analysis up to 15 samples per hour. Since the basis of the assay is sulfhydryl determination, it can be applicable for measurement of sulfhydryl utilization

for any enzymatic reaction that consumes or produces free sulfhydryl groups.

Automation of the GSHPx enzyme assay has been problematic. Most automated methods published do not yield equivalent results to the standard coupled assay [172,173]. A fully automated continuous-flow colorimetric method for GSHPx assay was reported in 1990 by Hawkes and Craig [174]. In this method GSH oxidation was monitored by its effect on the reaction of GSH with DCIP. Results of assays carried out by this method correlate well with the standard manual coupled assay; the assay works equally well with hydrogen or cumene peroxides as oxidants and shows the same selectivity toward GSTs as the standard assay. The automated method requires less work and is cheaper than the coupled assay, without sacrificing sensitivity, accuracy and precision. This method was applied for enzyme determination in whole blood, plasma, serum, and erythrocytes from human, rats, rabbits and monkeys.

Most of the reported assays for GST activity are continuous methods based on thioether or thioester formation [18,175–178]. The enzymatic reaction can be followed spectrophotometrically monitoring the direct change in absorbance of the substrate when it is conjugated with GSH. Different members of the GSTs family may be distinguished from each other by their reactivity towards several substrates [27,179,180]. Substrates such as: CDNB [181], *p*-nitrophenethyl bromide, *p*-nitrobenzyl chloride, 1,2-epoxy-3-[*p*-nitrophenoxy]propane, *trans*-4-phenyl-3-buten-2-one, ethacrynic acid [18], 1,2-dichloro-4-nitrobenzene [176], 1-menaphthyl sulfate [177] and Δ^5-androstene-3,17-dione [178], have been widely used to assay GST activity. In most cases detection is carried out in the UV region (wavelengths ranging 248–345 nm). With the exception of θ class isoenzymes, all other GSTs conjugate CDNB to GSH [182–184] and therefore this substrate is considered as "universal" for GSTs; while other substrates are more specific. Thus, ethacrynic acid is one of the better spectrophotometric substrates for acidic GST isoenzymes and lack substrate inhibition at high GSH concentration [185–187], bromosulfophthaleine and benzo[a]pyrene diol oxide, are specific towards the μ class, acroleine for the π class and 1,2-epoxy-3-(*p*-nitrophenoxy)-propane for the θ class [182,188]. Sometimes, the conjugation of these substrates with GSH is a spontaneous chemical process and therefore, control samples with no enzyme are needed to quantitate the enzymatic reaction. Iodomethane and alkyl halides have been used to assay GSTs by titrimetry [189]. Nitroalkanes and other organic nitro compounds [190,191] are also useful for assaying GST activity. Conjugation of these compounds with GSH releases nitrite, which can be determined by diazotization with sulfanilamide that quantitatively produces a colored compound [192].

A wide variety of structurally unrelated compounds have been shown to induce GST activity in a number of organisms, and differential induction of the isoenzymes has been reported [193,194]. Induction of a particular isoform by a given compound can be masked if only the overall GST activity of a crude extract is analyzed, hence the whole isoenzyme pattern needs to be determined. To this end, there are several methods which include an initial affinity chromatography step on GSH–Sepharose or S-hexyl GSH–Sepharose [62,89] followed by either CF [195], IEF [196], HAC [197] or RP-HPLC [63]. This last method is based on determination of the protein subunit and does not require preservation of activity, whereas the others are based on the quantitation of enzymatic activity. All rely on a similar initial step that uses an affinity matrix to which some of the isoenzymes do not bind or bind loosely, lowering recovery [63,194,195]. We have reported [198] a single-step analytical method based on ion-exchange HPLC with automatic on-line detection of activity using CDNB as substrate, for the rapid quantitation of GST isoenzymes in cell-free extracts. This method can be carried out in less than 3 h from the intact tissue to production of the isoenzyme activity chromatogram. It gives information on isoenzymes as they are in vivo and not on the subunit components artificially separated by the denaturing conditions of SDS-PAGE or RP-HPLC. The method has worked satisfactorily in our laboratory for the detection of differences in the levels of individual isoenzymes in hepatic extracts from fish captured in contaminated and clean waters. A schematic description of the HPLC set-up used for the on-line detection of GST activity is shown in Fig. 7. This system, with an autosampler and without the chromatographic column, can be used as a photometric flow injection

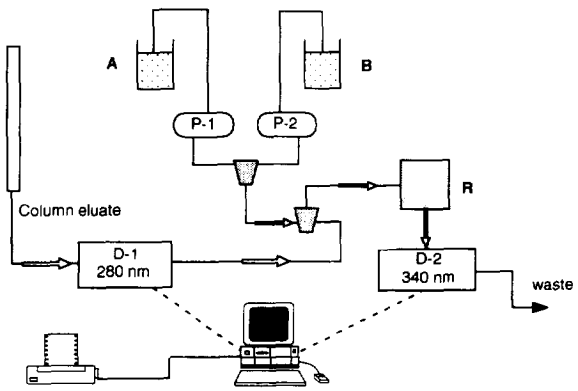

Fig. 7. Set-up of the on-line post-column detection system for GST activity. (A) CDNB solution; (B) GSH solution; (P-1 and P-2) HPLC pumps; (D-1) detector for protein set at 280 nm; (D-2) detector for GST activity set at 340 nm; (R) reactor coil in a water-bath at 40°C.

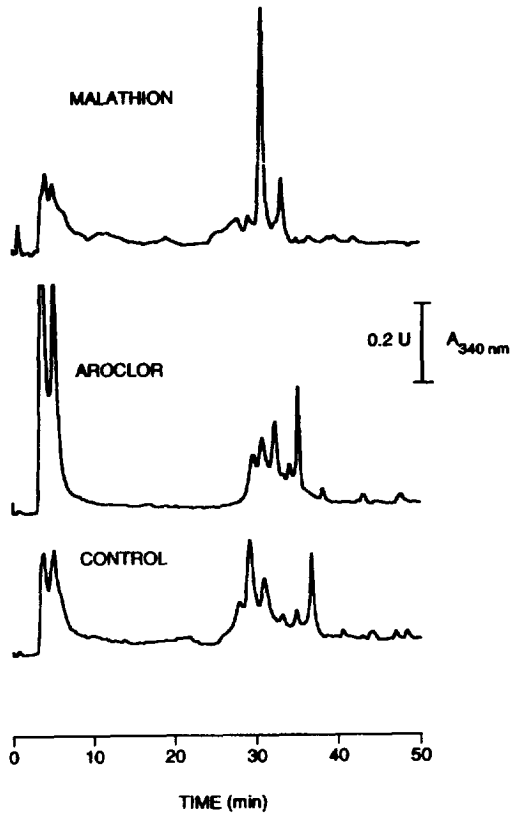

Fig. 8. GST activity profiles of hepatic soluble fraction from seabreams. Equipment, Beckman System Gold HPLC gradient system. GST isoenzymes were eluted from a DEAE Spherogel TSK column (7.5×0.75 cm I.D.) with a linear NaCl gradient (0–0.35 *M*) in 10 m*M* Tris–HCl buffer (pH 7.5), 1 m*M* EDTA, 5 m*M* GSH, 2 m*M* DTT, starting at 15 min and ending at 60 min at 1 ml/min. Post-column substrates were pumped at 1 ml/min. Monitorization was performed at 340 nm; cell-free extract samples (50–200 μl), containing up 15 mg/ml, can be analyzed with this set-up. The total GST activities injected into the column were 0.9, 0.8 and 0.96 units for fish exposed to malathion, Aroclor and corn oil (control), respectively.

analysis system (FIA) for the automated analysis of about 30 samples/h. Fig. 8 shows the GST isoenzyme activity profiles of hepatic soluble fractions from seabreams (*Sparus aurata*) exposed for two days to model xenobiotics. Labeled peaks correspond to identified subunits.

Most of the methods used to assay GSSGRase activity are based on either measuring the disappearance of NADPH or the appearance of GSH in the reaction mixture. The rate of oxidation of the pyridine nucleotide can be followed spectrophotometrically at 340 nm as described by Racker [199]. Since the reaction rate is considerably affected by temperature and concentration of reactants, many modifications of the original method have been published to achieve an optimal reaction rate [75,78,200–202]. Among the procedures described for the quantitation of GSH formed [203,204], the method described by Beutler and Yeh [205] using DTNB is the most widely used, and the reaction is followed spectrophotometrically at 412 nm.

4.2.2. Discontinuous assay methods

Some enzymatic reactions occur at a slow rate and, thus, require long times and sensitive detection to follow the reaction kinetics. In these cases, HPLC could be the method of choice to carry out the assay. This methodology offers the potential to simultaneously monitor several components during the course of the reaction, making it suitable for kinetic studies. New advances in robotic handling of samples, special devices to perform pre- and post-column derivatizations, premixing operations in autosamplers and new column technologies have made HPLC a very powerful separation technique to assay enzymatic activities in an high number of samples which can be processed overnight [206–210]. Differ-

ent strategies to design HPLC-based assays for many enzymes have been described by Rossomando [211–213] and recently by Lambeth and Muhonen [214].

Discontinuous methods using chromatographic techniques have been reported to assay GSHPx. Thus, a direct micro-assay for GSHPx based on the separation and quantitation of GSSG by RP-HPLC was reported by Xia et al. [215]. We have reported [216] a fast, sensitive and direct method for the determination of GSHPx activity (both Se and non-Se dependent) in cell-free preparations such as calf haemolysate, rat liver and fish liver. This assay is based on the separation and quantitation of GSH and GSSG by CE. The separation buffer was 100 m*M* sodium tetraborate pH 8.2 containing 100 m*M* SDS; a micellar electrokinetic mechanism took place under these conditions and a total mass recovery was observed for both peptides. The electropherogram shown in Fig. 9 corresponds to the direct assay of GSHPx using hydrogen peroxide as oxidant and calf haemolysate as enzyme source. The GSHPx activity was determined both with hydrogen peroxide which is highly selective for the selenium-dependent enzyme, and with cumene hydroperoxide, which is active with both the Se- and non-Se-dependent enzymes, thus measuring "total" GSHPx activity.

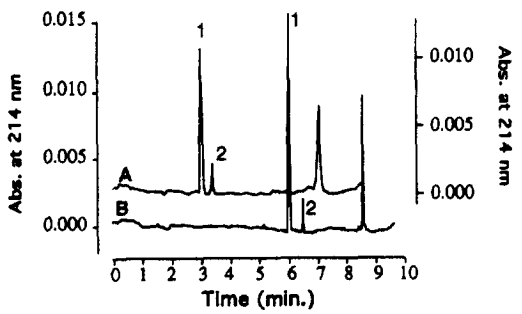

Fig. 9. Capillary electrophoresis migration pattern of GSH and GSSG. (A) Sample, 5 μl of rat liver extract in the assay mixture; oxidant, cumene hydroperoxide; reaction time, 3 min; capillary, 20 cm×50 μm I.D.; voltage, 8 kV (45 μA). (B) Sample, an assay mixture containing 3 μl of calf hemolysate and hydrogen peroxide as oxidant; reaction time, 2 min; capillary column, 50 cm×75 μm I.D.; voltage, 16 kV (130 μA). For both A and B: injection time, 3 s under the pressure mode; separation buffer, 100 m*M* sodium tetraborate, pH 8.2, 100 m*M* SDS; temperature, 30°C. Peaks: 1 = GSH, 2 = GSSG. Reprinted from Ref. [216] with permission from Elsevier, Oxford.

The results obtained with the direct CE assay were compared with those derived from RP-HPLC [215] and coupled spectrophotometric assay [162]. A very good agreement was found between the two direct assay methods in all samples. This CE assay is cheaper and less labour-intensive than the HPLC and coupled assays, with improved accuracy and precision. Using a 20-cm capillary column, it is possible to run up to 12 samples/h under an automatic mode, versus less than 8 samples/h with HPLC and 3–5 samples/h achieved with the photometric coupled assay.

GSHPx activity, can be also followed by monitoring the reduction product formed from the hydroperoxide. The separation and quantitation of hydroperoxides and their reduction products can be accomplished using HPLC [217] or TLC [217–219]. These methods are particularly useful to assay GSHPx activity towards membrane hydroperoxides, such as cholesterol and phosphatidyl choline hydroperoxides [217,218]. Visualization of hydroperoxide in TLC plates can be carried out using N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD) [219]. In the HPLC-based method described by Yamamoto et al. [217], the reaction products were extracted with chloroform–methanol, the extract dried and the residue dissolved in methanol. The separation was made isocratically on a TSK Silica-60 (25 cm × 4.6 mm I.D.) column using acetonitrile–methanol–water as eluent. Peak identification and quantitation of the reaction products was accomplished using the hydroxy derivatives formed by reduction of phosphatidyl choline with sodium borohydride as standard.

Chromatography has been also reported for assay of GST activity [96,220–222]. A TLC-based method to assay microsomal GSTs was reported in 1989 by McLellan et al. [96]. The substrate used was ^{14}C-radiolabeled hexachlorobuta-1,3-diene (HCBD). After incubation of samples for 1 h in a bath at 37°C with shaking, the enzymatic reaction was stopped in by cooling in ice and the sample was chromatographed using *n*-butanol–acetic acid–water (12:3:5, v/v) as running solvent on silica-coated polyester sheets. Detection was carried out using a liquid scintillation spectrometer. The use of HPLC to assay GSTs was initially reported in 1982 by Brown et al. [221]. The substrate used in this assay was styrene

oxide, and the enzymatic reaction was terminated by adding ice-cold ethyl acetate to extract the unreacted styrene oxide. Chromatography was carried out in a C_{18} μ-Bondapak column with isocratic elution, monitoring the eluate at 254 nm. This method was applied for assay of the GST activity in rat lung and liver cytosol samples.

HPLC is an useful technique for carrying out kinetic studies and Te Koppele et al. [222] have reported a HPLC assay to study the stereoselectivity of rat liver GSTs towards α-bromovaleric acid [BI] and α-bromovalerylurea [BIU] enantiomers. The analysis of conjugated GSH was carried out using RP-HPLC and electrochemical detection. This method allows the behaviour of GST isoenzymes towards these chiral substrates in isolated hepatocytes to be studied. The α-multigene GST family are the most important contributors in the GSH conjugation towards the (*S*)-enantiomer of BI, whereas the μ-family GSTs are predominant in the conjugation with BIU and are selective toward the (*R*)-enantiomer [222].

In the recent literature, discontinuous assay methods have been reported in about 4% of papers examined dealing with GSHPx and GSTs, being infrequently used to assay GSSGRase, probably because of the efficiency of the spectrophotometric assay available for this last enzyme.

5. Conclusions

To design a purification strategy maximizing purity, yield and economy, it is essential to take full advantage of the many separation technologies currently available. Among the different preparative techniques, chromatography has played an important role, being utilized in all reviewed papers. Recent advances on column technology and equipment are presented each year at the Pittsburg Conference. The high growth rate of chromatographic techniques in the last two decades has somewhat slowed down in recent years and it is generally accepted that more modest advances can be expected in the years to come. As evidence, in ca. 80% of protocols reviewed, the application of at least one chromatographic step using soft gel has been reported. Nevertheless, in our opinion, chromatography may have a brilliant future for protein separation. The goals of new method development are to save time and sample. A speculative look towards future developments suggests that many exciting innovations, such as "molecular imprinting" [223,224] and "hyperdiffusion" chromatography [225–227], will contribute, in the years to come, to very fast separation of complex protein mixtures with almost quantitative recovery of activity. Nowadays, CE is a powerful tool for solving separation problems, however, we expect that further advances in capillary coating and detection systems are needed to make this methodology a powerful technique for routine work. The integration of these new methodologies in analytical and preparative separation schemes will be a challenge in the near future.

6. List of abbreviations

AC	affinity chromatography
2′,5′-ADP	adenosine-2′,5′-diphosphate
BSA	bovine serum albumin
CDNB	1-chloro- 2,4-dinitrobenzene
CE	capillary electrophoresis
CF	chromatofocusing
CM	carboxymethyl
C8ATPR	8-[6-aminohexyl]-amino-2′-phosphoadenosine-5′- diphosphoribose
DEAE	diethylaminoethyl
DCIP	2,6-dichlorophenolindophenol
DTNB	5,5′-dithio-bis-[2-nitrobenzoic acid]
DTT	dithiotreitol
FPLC	fast protein liquid chromatography
GSH	glutathione
GSHPx	glutathione peroxidase
GSSG	oxidized glutathione
GSSGRase	glutathione reductase
GST	glutathione transferase
HAC	hydroxyapatite chromatography
HCBD	hexachlorobuta-1,3-diene
HIC	hydrophobic interaction chromatography
HPLAC	high-performance liquid affinity chromatography
HPLC	high-performance liquid chromatography
IEC	ion-exchange chromatography

IEF	isoelectric focusing
$NADP^+(H)$	nicotinamide adenine dinucleotide phosphate and its reduced form
PAGE	polyacrylamide gel electrophoresis
PEEK	polyetheretherketone
PEI	polyethyleneimine
PMSF	phenylmethanesulfonyl fluoride
QA	quaternary ammonium
RP-HPLC	reversed-phase HPLC
SEC	size-exclusion chromatography
SDS	sodium dodecyl sulfate
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SP	sulfopropyl
TLC	thin-layer chromatography
TMPD	N,N,N′,N′-tetramethyl-*p*-phenylenediamine

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