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Characterization of Glutathione *S*-Transferase Activity in the Asiatic Clam *Corbicula fluminea*

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The glutathione *S*-transferases (GSTs; EC 2.5.1.18) form a group of multifunctional enzymes catalyzing the conjugation of a broad range of electrophilic substrates to the tripeptide glutathione (Wilce and Parker 1994). They are involved in the cellular detoxification and excretion of many physiological and xenobiotic substances. Moreover, cells may respond to xenobiotic exposure by induction of particular isoenzymes (Ketterer et al. 1988). They have been mainly studied in mammals (Wilce and Parker 1994) but numerous studies also report their presence in invertebrates (Dierickx 1984, Stenersen et al. 1987, Livingstone 1991), yeasts (Foley and Sheehan 1998) and plants (Schrenk et al. 1998). Because GSTs can be induced or inhibited by certain xenobiotics, they have been proposed as potential pollution biomarkers for several aquatic species such as fishes, crustaceans or mollusks. Thus, increase of GST activity as a result of exposure to certain compounds has already been demonstrated in these species (Lee et al. 1988; Boryslawskyj et al. 1988; Tejo Prakash and Jagannatha Rao 1995).

Corbicula fluminea is a freshwater bivalve originating from Asia that has become a major component of many benthic communities in the United States and more recently in Europe (Araujo et al. 1993). It is known to accumulate pollutants such as heavy metals (Baudrimont et al. 1997) or polycyclic aromatic hydrocarbons (Narbonne et al. 1999). In our laboratory we study the possible use of this species as a sentinel organism of freshwater ecosystems. As we previously observed the presence of GST activity in *C. fluminea*, it must be now characterized in order to include it in a multibiomarkers profile. As a preliminary study of purification procedure, this paper reports GST enzymatic characterization and assay optimization on crude fractions using several substrates.

MATERIALS AND METHODS

Reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid (EA), 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) and 3,4-dichloro-1-nitrobenzene (DCNB) were obtained from Sigma-Aldrich Chemical, Saint Quentin Fallavier, France. Other chemicals were of the best technical grade available.

Adult *Corbicula fluminea*, anterioposterior length of which ranging from 15 to 20 mm, were collected during the spring 1998 from the banks of the non-polluted freshwater Cazaux-Sanguinet lake (Aquitaine, France). They were placed into a plastic tank containing lake water for the transport back to the laboratory. They were maintained in stabulation for seven days prior the experiment in glass aquariums containing dechlorinated tap water. Temperature was maintained at 18.5 °C and aeration was provided by air bubbling. Clams were not fed during this period and were held under a natural light cycle. No sexual differences were taken in account for *C. fluminea* are

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hermaphroditic.

All steps of homogenization were achieved at 4°C. After the shell and the crystalline style were removed, gill, foot, visceral mass, and mantle were dissected-out from a pool of 100 animals. Bodies samples were rinsed in 100 m*M* phosphate buffer, pH 7.4, dried on absorbent paper sheets, weighed and homogenized in the same phosphate buffer (1:4 weight:volume ratio) using an Ultra-Turrax® Antrieb T25 (IKA® Labortechnik). Homogenates were then centrifuged at 9000 g for 30 min in a Sigma 3 MK centrifuge. Parts of the supernatants, consisting in the submitochondrial fractions (S9), were collected and stored at -80°C. Microsomes were obtained by centrifuging fractions of the S9 set apart at 105000 g for 1 hr in a Beckman LE-80 ultracentrifuge. The supernatants (cytosolic fractions) were collected and stored at -80°C. Microsomal pellets were resuspended in a 100 m*M* phosphate buffer, pH 7.4, containing 1 m*M* EDTA and 20 % glycerol, then collected and stored at -80°C.

GST activities were measured on a temperature-controlled Kontron Uvikon 932 spectrophotometer. Assays were run in triplicate. Four substrates were assayed (CDNB, EA, EPNP, DCNB) according to the method of Habig et al. (1974) following the apparition of glutathione conjugates at 340 nm (CDNB), 270 nm (EA), 360 nm (EPNP) and 345 nm (DCNB). A 100 m*M* phosphate buffer pH 7.4, a sample protein concentration of 0.1 mg/mL and a temperature of 25 °C were used for all substrates as initial standard assay conditions. Particular assay conditions for each substrate were as follows. CDNB : 1 m*M* GSH and 1 m*M* CDNB; EA : 0.25 m*M* GSH and 0.2 m*M* EA; EPNP : 5 m*M* GSH and 0.5 m*M* EPNP; DCNB : 5 m*M* GSH and 1 m*M* DCNB. Total assay volume was of 1 mL. As we progressed in the study, optimal parameters were determined for each substrate. CDNB, EA, EPNP and DCNB were diluted in ethanol. The concentration of this latter did not exceed 2% in assay. GSH was diluted in phosphate buffer. Sample concentration, buffer pH, buffer concentration, temperature and substrate concentration varied in different experiments, details of which are described in relevant sections of the "Results and Discussion". GST activities were expressed as specific activities (in nmoles/min/mg protein) or as reaction rates (nmoles/min). The enzymatic rate was obtained by subtracting the chemical rate (without sample) to the total rate. Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Assuming Michaelis-Menten kinetics, apparent K_m and V_{max}, values of the GST activities were calculated using the computer program Statistica (5.1 release, Statsoft®). In a reaction rate vs. substrate plot this program fitted a non-linear regression curve by using least squares for minimizing the deviations in reaction rate on the given substrate-values. For each set of observations, the program presented the values for the measured and calculated reaction rate and finally it provided the apparent K_m and V_{max} values as calculated from the fitted curve.

RESULTS AND DISCUSSION

In *Corbicula fluminea*, the subcellular distribution of GST activities was quite similar using CDNB or EA as substrates (cf. table 1). They were mainly localized in cytosolic fractions as expected (53 % and 52 % for CDNB and EA respectively) for most of GST isoenzymes are cytosolic ones. They were localized in a minor extent in submitochondrial ones (40 % and 38 % for CDNB and EA respectively). Low but substantial GST activity towards CDNB was also observed in microsomal fractions (7 %) and could not only be explained by a contamination of these fractions by submitochondrial particles. Such an activity was also observed in microsomal fraction of hepatopancreas of the freshwater crayfish *Astacus astacus* (Lindström-Seppä et al. 1983).

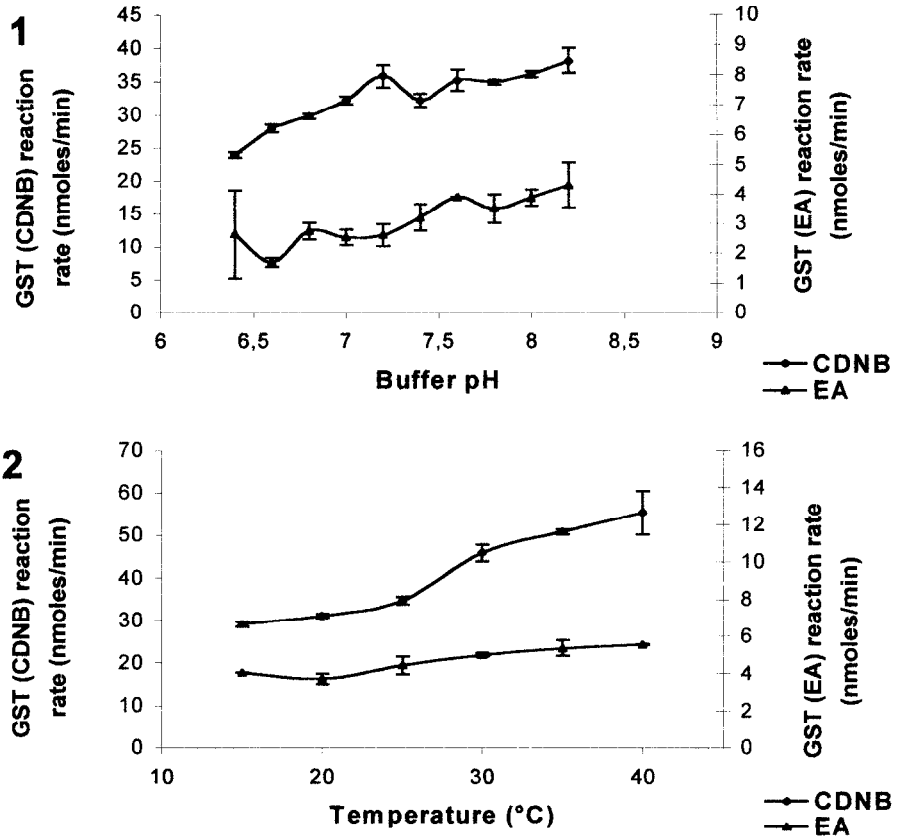
Table 1 . Subcellular and tissular distributions of GST specific activities towards CDNB and EA. Results are expressed in nmoles/min/mg protein. Values are means ± SD (n=3).

Substrate / Fraction	Tissue			
	Foot	Gill	Mantle	Visceral mass
CDNB				
Submitochondrial	53.7 (± 1.5)	149.8 (± 8.1)	107.5 (± 2.7)	554.7 (± 4.8)
Cytosolic	98.5 (± 1.6)	206.9 (± 4.2)	118.8 (± 5.8)	720.9 (± 10.5)
Microsomal	18.7 (± 1.4)	20.7 (± 0.3)	27.5 (± 2.2)	85.9 (± 2.9)
EA				
Submitochondrial	18.5 (± 1.6)	32.2 (± 3.4)	30.6 (± 4.8)	54.7 (± 1.6)
Cytosolic	32.1 (± 4.1)	45.7 (± 1.8)	35.7 (± 4.1)	71.0 (± 1.2)
Microsomal	3.7 (± 3.7)	6.9 (± 0.4)	8.2 (± 3.2)	15.2 (± 3.7)

Regarding tissue distribution, GST activity of *C. fluminea* towards CDNB was found to be ubiquitous in tissular fractions as already observed for *Mytilus edulis* or *A. astacus* (Fitzpatrick and Sheehan 1993 ; Lindström-Seppä et al. 1983). It was predominant in visceral mass and in a lesser extent in gill (63 % and 18 % respectively in cytosolic fractions) whereas in the case of *M. edulis,* it was mainly localized in the gill (Fitzpatrick and Sheehan 1993). In mollusks, digestive gland presents functions analogous to that of vertebrate liver in which high GST activity is encountered. The visceral mass of C. *fluminea* includes two organs impossible to dissociate without breaking their integrity, namely the digestive gland and gonads. Thus the high activity observed in the visceral mass could be considered as the sum of activities of both organs. *C. fluminea* also exhibited GST activities towards more specific substrates as EA, EPNP and DCNB but they were lower than that observed with CDNB (results not shown for EPNP and DNCB). When using EA as a substrate *C. fluminea* presented a relatively high activity (up to 10 % of activity towards CDNB) and the tissular distribution was close to that observed for CDNB (39 % and 25 % in cytosol of visceral mass and gill respectively). In the study of Stenersen et al. (1987) *M. edulis* exhibited one of the greatest GST activity towards EA among aquatic and terrestrial animals studied. We observed GST activity towards EPNP in almost every subcellular and tissular fraction but at a low level (4 % of that with CDNB in cytosol of visceral mass). Moreover it exhibited a high variability that could not be reduced despite steps of assay optimization. Such a variability was already observed in the yeast (Foley and Sheehan 1998). Among invertebrates, activity towards EPNP was neither detected in the purified isoenzyme GST 1 of *M. edulis* (Fitzpatrick et al. 1995) nor in several species of earthworms (Stenersen et al. 1979). In the case of DCNB, GST activity was not observed in cytosolic fractions of *C. fluminea.* As it was assayed on cytosolic crude fractions, it was perhaps too low to be detected. Thus, Stenersen et al. (1987) did not observe activity in most of invertebrates studied *(M. edulis* included) whereas it was detected in the purified cytosolic isoenzyme GST 1 of *M. edulis* gill (Fitzpatrick et al. 1995). On the other hand, we detected activity in microsomes of visceral mass (3.06 nmoles/min/mg protein ± 1.01). For the following studies, GST activities were measured in cytosol of visceral mass using CDNB and EA as substrates.

Under standard assay conditions, GST reaction rate was found to be linear with protein concentration in the range from 0.01 to 0.15 mg protein/mL for CDNB and from 0.01 to 0.2 mg protein/mL for EA (results not shown). For the following steps of the study, a protein concentration of 0.05 mg protein/mL was selected for both substrates.

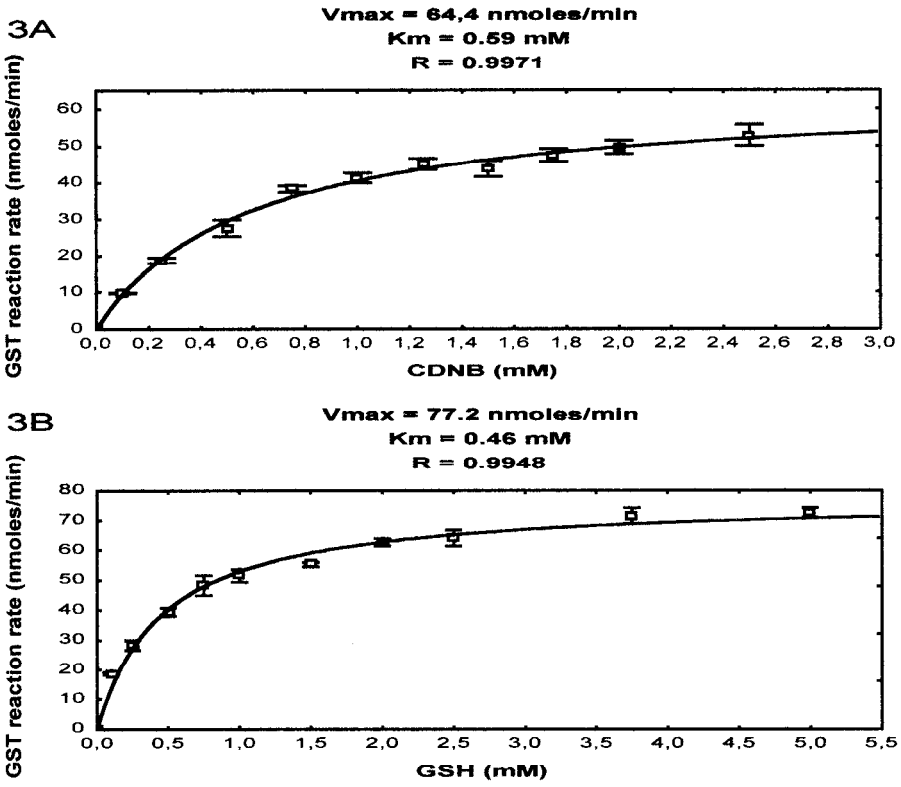
To study the dependence of GST reaction rate on buffer pH, a range of phosphate buffer pH from 6.4 to 8.2 was assayed. The presence of at least two pH optima for CDNB and EA conjugation was observed for *C. fluminea* (pH 7.2 and 7.6 for CDNB, pH 6.8 and 7.6



Figures 1 & 2 . Dependence of GST reaction rate on buffer pH (figure 1) or temperature (figure 2) using CDNB or EA as a substrate. Values are means ± SD (n=3).

for EA) (cf. figure 1). Such results let us foretell for the possible presence of at least two major GST isoenzymes in the cytosol of visceral mass. Few studies are available on the pH optimum of GST activities among invertebrates. Balabaskaran et al. (1986) observed pH optima varying between pH 7 and 9 for CDNB conjugation in three gastropod species. More generally, pH optima with a variety of substrates range from 6.5 to 9.5 for non-vertebrate organisms with an optimum in the vicinity of pH 8 for CDNB (Clark 1989). In the same review, the author reported that the distribution of pH optima for CDNB could be bimodal, indicating the possible existence of two general classes of enzyme. Three buffer concentrations were also evaluated : 25, 50 and 100 m*M*. A maximal GST reaction rate was obtained for a concentration of 50 and 25 m*M* using CDNB and EA respectively (results not shown). For further studies, we selected a 50 m*M* buffer, pH 7.2 and a 25 m*M* buffer, pH 7.6 in the case of CDNB and EA respectively.

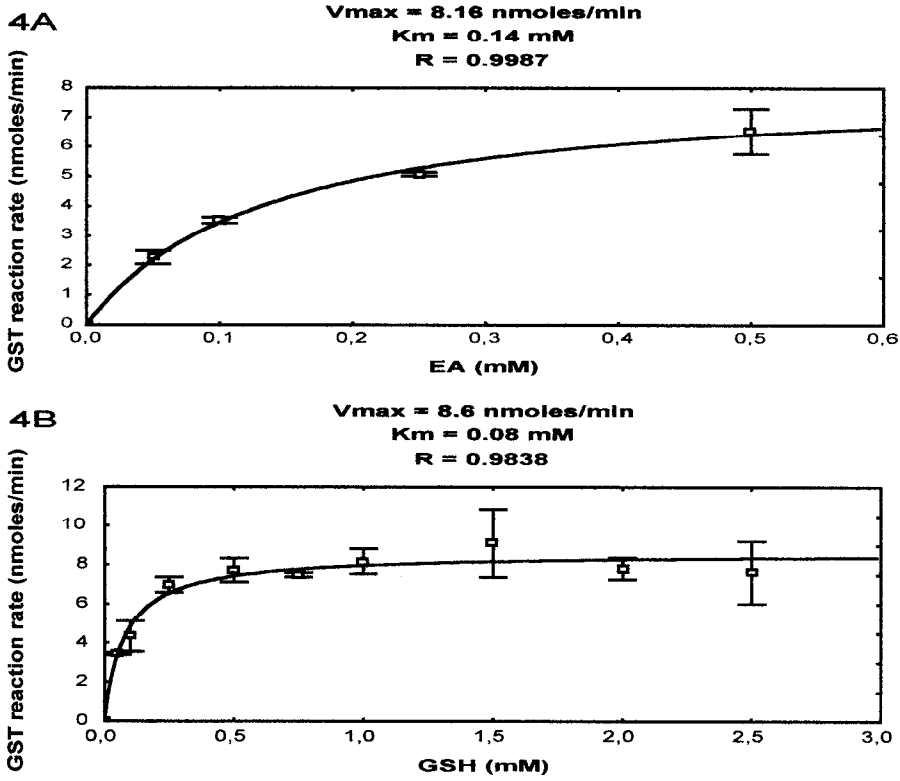
To study the dependence of GST reaction rate on temperature, a range of temperatures from 15 to 40°C was assayed. Using CDNB as a substrate, the reaction rate increased along with temperature with an inflexion between 25 and 30°C (cf. figure 2). In the case of EA, it slightly decreased between 15 and 20°C and then increased slowly up to 40°C. Whatever the substrate, no denaturation point was detected in the range assayed. Arrhenius graphs obtained for CDNB and EA were discontinuous (results not shown) as already



Figures 3A & 3B . Dependence of GST reaction rate on CDNB (figure 3A) and GSH (figure 3B) concentrations. Apparent kinetic parameters and regression coefficient of the Michaelis-Menten model fitted curve are given for each substrate.

observed among insects (Clark 1989). It could be due to the presence of several isoenzymes in the crude extract. Activation energy of *C. fluminea* GST using CDNB was close to these observed in other non-vertebrate species as reported by Clark (1989), namely in the vicinity of 30 Kj/mole (26 Kj/mole for *C. fluminea* above 25°C). That obtained for EA was dramatically lower (1.4 KJ/mole above 20°C) suggesting a different mechanism of conjugation with this substrate. A temperature of 30°C for CDNB and EA was selected for the following assays.

Results of dependence of the reaction rate on the substrate concentration are presented on figures 3A and 4A. For the determination of kinetic parameters, only substrate concentration varied. Ethanol concentration was maintained constant (2%). Other parameters were as determined before. Using CDNB, ten concentrations of this substrate were assayed, ranging from 0.1 to 2.5 m*M*. We were limited for the choice of the upper concentration in that CDNB solubility in assay did not exceed 2.5 m*M* at 30°C. When using EA, eight concentrations were assayed ranging from 0.05 to 2 m*M*. No problem of solubility in the assay was encountered. Curves of GST reaction rate vs. substrate concentration fitted the Michaelis-Menten model with a regression coefficient R of 0.997 and 0.998 for CDNB (despite slightly discontinuities) and EA respectively. In the case of EA, only four plots were used for kinetic parameters determination as GST reaction rate rapidly decreased from a substrate concentration located between 0.5 and 0.75 m*M* (cf. figure 5). No more activity was detected from a concentration of 1 .5 m*M*. The same effect



Figures 4A & 4B . Dependence of GST reaction rate on EA (figure 4A) and GSH (figure 4B) concentrations. Apparent kinetic parameters and regression coefficient of the Michaelis-Menten model fitted curve are given for each substrate.

was observed when using a higher GSH concentration. EA and especially its GSH-conjugate are known as inhibitors of GSTs in humans using CDNB as a substrate (Ploemen et al. 1993). The same phenomenon seems also occur in *C. fluminea* except that in our case, EA is both substrate and inhibitor. Apparent K_m, of GST activity towards CDNB (0.59 m*M)* was higher than that of EA (0.14 m*M)* suggesting an higher affinity of GST for EA than for CDNB. For following studies using CDNB, we could not be at the V_{max} because of the limited solubility of this substrate. Nevertheless, we selected a CDNB concentration of 1 .75 m*M* higher than the apparent K_m In the case of EA, a concentration of 0.5 m*M* was selected.

Results of dependence of the reaction rate on the GSH concentration are presented on figures 3B and 4B. For the determination of kinetic parameters, only GSH concentration varied. Other parameters were as determined before. Using CDNB, ten concentrations of GSH were assayed, ranging from 0.1 to 5 m*M* whereas for EA, eight concentrations of GSH were assayed, ranging from 0.05 to 2.5 m*M*. Despite discontinuities, curves of GST reaction rate vs. GSH concentration fitted the Michaelis-Menten model with a regression coefficient R of 0.995 and 0.984 for CDNB and EA respectively. Apparent K_m^{GSH} *using* CDNB as a substrate was higher than that observed with EA suggesting an higher affinity of GST using EA for GSH than GST using CDNB *(K_m^{GSH}* of 0.46 m*M* and 0.08 m*M* for CDNB and EA respectively). As a general manner, GST activity had an higher affinity for GSH than for the conjugating substrate. Thus, GST in *Corbicula* seems to be constituted

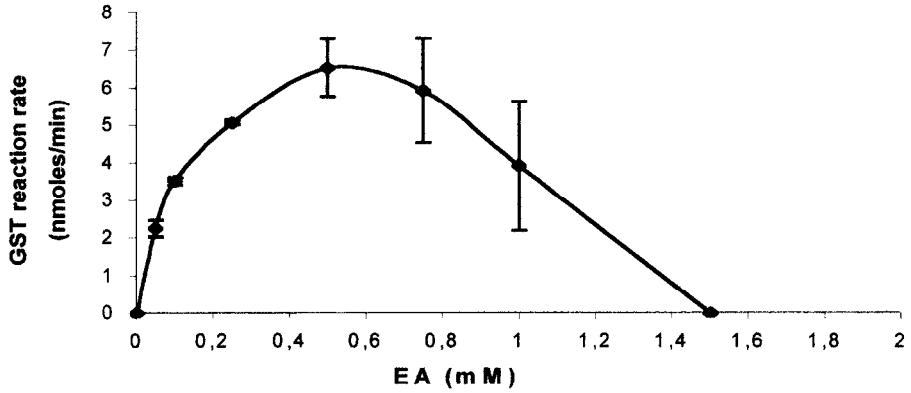


Figure 5. Dependence of GST reaction rate on EA concentration. Values are means ± SD (n=3).

by a very specific GSH binding site and a less specific electrophilic substrate one as it is the case in mammals (Wilce and Parker 1994).

It is difficult to compare these apparent kinetic parameters with those found in literature in different assay conditions. Nevertheless, our values are close of those obtained by Keeran and Lee (1987) for two purified isoenzymes of the blue crab *Callinectes sapidus* (K_m^{CDNB} of 0.28 and 0.31 m*M*, K_m^{GSH} of 0.14 and 0.43 m*M* for isoenzyme I and II respectively). For the purified isoenzyme GST 1 of *M. edulis* gill, K_m^{CDNB} (3.7 m*M)* was much higher whereas K_m^{GSH} *(0.5 mM)* was close of that observed for *C. fluminea* (Fitzpatrick et al. 1995). The slight discontinuities observed on curves of GST reaction rate vs. CDNB or GSH concentration could be explained by the possible presence of several isoenzymes in the crude fractions, as hypothesized before. For further assays, GSH concentrations of 5 m*M* and 0.25 m*M* for CDNB and EA respectively were selected to be close of V_{max} conditions.

As other mollusks, cytosolic fractions of *C. fluminea* exhibit high GST activity towards CDNB and in a lesser extent, EA. Several isoenzymes may be present and must be further characterized.

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