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Oesophagostomum dentatum: Effect of glutathione *S*-transferase (GST) inhibitors on GST activity and larval development

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ABSTRACT

Sulphobromophthalein (SBP) inhibits isolated glutathione *S*-transferase of the porcine nodule worm *Oesophagostomum dentatum* (Od-GST) and reduces larval development in vitro. In this study possible inhibitory effects of various inhibitors were evaluated in an enzymatic (CDNB) assay with isolated Od-GST and in a larval development assay (LDA). Reversibility was tested in the LDA by removing the inhibitor from culture halfway through the cultivation period. SBP, indomethacin and ethacrynic acid inhibited both enzyme activity and larval development in a dose-dependent and reversible manner. HQL-79 also reduced larval development but had only a minor effect on the isolated enzyme. The phospholipase A_2 inhibitors dexamethasone and hydrocortisone had no major effect. High thermal stability of Od-GST was demonstrated with increasing activity between 4 and 50 °C. Differences between Od-GST and GST of other organisms indicate structural and possibly functional peculiarities and highlight the potential of such enzymes as targets of intervention.

1. Introduction

Glutathione *S*-transferases (GSTs) have been implied in a variety of functions in eukaryotic organisms (for review see Vermeulen et al., 1996), including cellular detoxification and homeostasis (Brophy and Barrett, 1990; Leiers et al., 2003; Angelucci et al., 2005; Jancova et al., 2010). They are also considered as possible drug targets for anthelminthic intervention (Barrett, 1995) or vaccination in filarial nematodes (Brophy and Pritchard, 1994; Rao et al., 2000; Campbell et al., 2001a; Gupta and Srivastava, 2006; Rathaur et al., 2008; Veerapathran et al., 2009) and hookworms (Xiao et al., 2008; Zhan et al., 2010); however, little is known about the biochemical properties and the biological function of GST in gastrointestinal nematodes. For the porcine nodule worm, *Oesophagostomum dentatum*, two isotypes of GST have been characterised on the mRNA and protein levels and constitutive expression of mRNA was demonstrated in all stages of the nematode, indicating housekeeping functions for the worm itself (Joachim and Ruttkowski, 2008). Addition of the GST inhibitor sulphobromophthalein (SBP) to in vitro cultures of *O. dentatum* reversely inhibited the development of third-stage larvae (L3) to fourth-stage larvae (L4). Based on the similarity of GST from *O. dentatum* with a synthetic prostaglandin D synthase (PGDS) (Joachim and Ruttkowski, 2008) the ability of native Od-GST to convert prostaglandin (PG) H_2 into PGD_2 was investigated further (Joachim and Ruttkowski, 2010). The finding that Od-GST can act as PGDS supports the important intrinsic role of GST for the nematode, since PGD and other eicosanoids have previously been demonstrated in homogenates and supernatants of *O. dentatum* larvae and were shown to be important for their ecdysis, development and motility (Daugschies, 1996; Daugschies and Ruttkowski, 1998; Joachim et al., 2005).

This work was conducted to investigate the influence of GST and PGDS inhibitors on the activity of purified native Od-GST and, in parallel, on the development of *O. dentatum* larvae in vitro.

2. Materials and methods

2.1. Parasite material

Third-stage larvae (L3) of *O. dentatum* (strain Hann-1) were derived from standard coproculture, kept at 11 °C for a maximum of 3 months, cleaned by micro-agar-gel migration and exsheathed by sodium-hypochlorite immediately before use as described previously (Joachim et al., 1997). Fourth-stage larvae (L4) and adult females and males were retrieved from the large intestines of pigs experimentally infected with 25,000 L3 12 days prior to slaughter

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(for L4) or patently infected (for adults) as described (Slotved et al., 1996). Each batch of worms isolated from the intestines was washed five times in 250 ml of 0.9% sodium chloride at 37.5 °C. After the final washing step the specimen were transferred to 2 ml cryotubes, most of the liquid was removed with a glass pasteur pipet and the worms were immediately snap frozen in liquid nitrogen. Animal experimentation was conducted in accordance with national Animal Welfare Regulations and approved by the Animal Ethics Committee.

2.2. Larval cultivation

L3 were counted, exsheathed and seeded into 24-well plates containing 2 ml of larval medium (Joachim and Ruttkowski, 2008) in portions of approximately 100 larvae/well. The actual number of larvae in each well was determined on the day of seeding. Plates were incubated at 38.5 °C and 10% CO_2 for 15 days (with a change of medium after 1 week). The percentage of developing L4 was determined on days 8 and 15 and calculated as the percentage of the larvae on day 1. Each inhibitor and the controls were run in quadruplicates unless indicated otherwise (see Section 3). Solvent controls were used for each assay. To determine the maximum concentrations of ethanol and DMSO tolerated by the larvae separate assays were performed applying ethanol (1% or 5%) or DMSO (0.01% or 0.1%) to the cultures. To test for reversibility of inhibiting effects larvae were cultivated with substances in concentrations that had previously been shown to have an inhibitory effect of approximately 50% (IC_{50}) or higher for 1 week, then the medium of half of the cultures was replaced by medium without inhibitor (termed "reverse") by three consecutive washing steps in inhibitor-free medium and cultivated until day 15.

2.3. GST preparation

Exsheathed L3, L4 and adult females and males were snap frozen immediately after harvesting, homogenised mechanically under liquid nitrogen as described (Joachim and Ruttkowski, 2008) and dissolved in 2 ml Binding Buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), centrifuged for 15 min at 10,000*g* at 4 °C and the supernatant was collected and filtrated with PVDF syringe filters, pore size 0.22 µm (Roth GmbH u. Co. KG, Karlsruhe, Germany). GST was extracted from the supernatant using glutathione columns (GSTrap HP®, GE Healthcare, Vienna, Austria) eluted with Elution buffer (50 mM Tris base and 10 mM reduced glutathione, pH 8.3). Eluates were concentrated to 250 µl on Amicon Ultra-4 centrifugal filter units, cut-off: 3 kDa (Millipore, Vienna, Austria) for at 7500 *g*. The solution was stored at −80 °C until use.

2.4. GST activity assay

Cytosolic fractions of GST were prepared and the enzyme concentration was determined as described (Joachim and Ruttkowski, 2008).

To determine GST activity a commercial Glutathione *S*-transferase Assay kit (Cayman Chemical Company, Ann Arbor, USA) was used at 25 °C as described by the manufacturer.

2.5. Inhibitors

Chemical GST inhibitors sulphobromophthalein (SBP), ethacrynic acid (ETA), the phospholipase A2 inhibitors dexamethasone (DEX) and hydrocortisone (HYC), the cyclooxygenase-2 inhibitor indomethacin (IND), and the prostaglandin D synthase inhibitor 4-benzhydryloxy-1-[3-(1H-tetrazol-5-yl)-propyl]piperidine (HQL-79, in DMSO or ethanol) were applied in different concentrations (Table 1). The maximum concentration of ethanol that could be added to the larval cultures without changes in larval development compared to an ethanol-free control was less than 5% and thus constituted the upper limit for the respective inhibitors (see Section 3).

Table 1
Final concentrations of inhibitors used in the CDNB assay or the bioassay. L3, L4: third-, fourth-stage larvae, F: females, M: males. Manufacturers: SBP, ETA, DEX, IND: Sigma Aldrich GmbH, Steinheim, Germany, HQL-79: Cayman Chemical Company, Ann Arbor, USA.

Substance	Solvent (highest concentration)	Final concentrations in CDNB assay (*stages tested*)	Final concentrations in bioassay
Sulphobromophtalein (SBP)	Assay buffer	15, 150, 1500 µM (*L3*)	15, 150, 1500 µM
Etacrynic acid (ETA)	EtOH (1%)	0.05, 0.5, 5, 10 µM (*L3*, *F*, *M*)	30, 100, 300, 1000 µM
Hydrocortisone ⩾98% (HYC)	EtOH (2.5%)	345, 172.5 µM (*L3*)	0.69, 6.9, 69 µM
Dexamethasone ⩾97% (DEX)	EtOH (1%)	n.d.[a]	0.255, 2.55, 25.5 mM
Indomethacin 99% TLC (IND)	EtOH (2.5%)	28, 56, 280 µM (*L3*, *L4*, *F*, *M*)	12.7, 127, 635, 1270 µM
HQL-79 DMSO	DMSO	1, 5, 10 µM (*L3*)	n.d.[a]
HQL-79 EtOH	EtOH (3.78%)	165, 330 µM (*L3*, *L4*, *F*, *M*)	2.5, 25, 250 µM

[a] Not done due to technical reasons (solvent concentrations too high for the respective assay).

The influence of thermal treatment on GST activity was determined by incubating isolated GST in a volume of 40–60 µl at different temperatures, placing it on ice afterwards and measuring the activity as described above. Tests were performed in the following series: (1) Incubation of L3 GST at 4, 10, 20, 30, 40 and 50 °C for 3 min (low temperatures); (2) Incubation of GST from L3, females and males at 4, 50, 60, 70, 80 and 90 °C for 3 min (high temperatures) using horse liver GST provided with the GST Assay as control at 70, 80 and 90 °C.

2.6. Statistical analysis

Correlations between inhibitor concentrations and enzyme activity, between inhibitor concentrations and larval development and incubation temperature and enzyme activity were calculated as Spearman's rank correlation coefficients.

3. Results

3.1. Activity of isolated GST

3.1.1. Thermal stability

Activity of GST from L3 increased with temperature until 50 °C; 70–90 °C incubation lead to decreasing inactivation with the GST from male worms showing the highest stability. The activity between 10 and 50 °C was positively correlated ($\rho = 0.71$) while between 50 and 90 °C the temperature increase correlated negatively with GST activity ($\rho = -0.92$–0.97 for the different developmental stages). Horse liver GST was completely inactivated at 70 °C for 3 min (Table 2).

3.1.2. Chemical inhibition of isolated GST

SBP, IND and ETA could inhibit GST activity in the tested stages (see Table 1) in a dose-dependent manner (Table 3), while HYC did not. HQL-79 DMSO could not inhibit GST activities while HQL-79 EtOH showed a slight but dose-dependent inhibition in all tested