Co-expression of glutathione S-transferase with methionine aminopeptidase: a system of producing enriched N-terminal processed proteins in *Escherichia coli*

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We describe here an *Escherichia coli* expression system that produces recombinant proteins enriched in the N-terminal processed form, by using glutathione S-transferase cGSTM1-1 and rGSTT1-1 as models, where c and r refer to chick and rat respectively. Approximately 90 % of the cGSTM1-1 or rGSTT1-1 overexpressed in *E. coli* under the control of a *phoA* promoter retained the initiator methionine residue that was absent from the mature isoenzymes isolated from tissues. The amount of initiator methionine was decreased to 40 % of the expressed cGSTM1-1 when the isoenzyme was co-expressed with an exogenous methionine aminopeptidase gene under the control of a separate *phoA* promoter. The recombinant proteins expressed were mainly methionine aminopeptidase. The yield of cGSTM1-1 was decreased to 10 % of that expressed in the absence of the exogenous methionine aminopeptidase gene. By replacing the *phoA* with its natural promoter, the expression of methionine aminopeptidase decreased drastically. The yield of the co-expressed cGSTM1-1 was approx. 60 % of that in the absence of

the exogenous methionine aminopeptidase gene; approx. 65 % of the initiator methionine residues were removed from the enzyme. Under similar conditions, N-terminal processing was observed in approx. 70 % of the recombinant rGSTT1-1 expressed. By increasing the concentration of phosphate in the growth medium, the amount of initiator methionine on cGSTM1-1 was decreased to 14 % of the overexpressed isoenzymes, whereas no further improvement could be observed for rGSTT1-1. The initiator methionine residue does not affect the enzymic activities of either cGSTM1-1 or rGSTT1-1. However, the epoxidase activity and the 4-nitrobenzyl chloride-conjugating activity of the purified recombinant rGSTT1-1 are markedly higher than those reported recently for the same isoenzyme isolated from rat livers.

Key words: mass spectrometry, protein maturation, rGSTT1 purification.

INTRODUCTION

Polypeptides synthesized on ribosomes are initiated with either a methionine or a formylmethionine residue. This residue is often deleted from the N-terminus of the mature protein [1]. In prokaryotes the initiator methionine can be deformylated and the leader methionine can then be cleaved from the proteins, depending on the identity of the penultimate amino acid residues. This N-terminal processing of proteins is performed by a ubiquitous enzyme called methionine aminopeptidase (Met-AP; EC 3.4.11.18) that has been cloned from both prokaryotic and eukaryotic organisms [2–6]. This enzyme favours substrates that possess a physically small residue situated adjacent to the initiator methionine residue. Studies on the expression of mutant forms of iso-1-cytochrome *c* in yeast [7] and methionyl-tRNA synthetase in *Escherichia coli* [8] indicate that initiator methionines can be effectively removed from proteins that have glycine, alanine, serine or threonine as penultimate residues. Proteins with valine and cysteine as penultimate residues can only be partly processed. Therefore a common and sometimes major problem in the heterologous expression of recombinant proteins in *E. coli* is the retention of an initiator methionine residue that should be cleaved [9].

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a group of isoenzymes involved in cellular detoxification by conjugating glutathione with a large variety of xenobiotics [10]. The cytosolic form of these isoenzymes can be grouped into six classes called Alpha, Mu, Pi, Theta, Sigma and Kappa [11–14]. Numerous structural and functional studies have been performed with these isoenzymes. The three-dimensional structures of GSTs of classes Alpha [15], Mu [16,17], Pi [18–20], Theta [21] and Sigma [22,23] have been elucidated. Except for earlier works on class Pi GSTs [19,20], the structures of these enzymes were determined on proteins heterologously expressed in *E. coli*. It should be noted that results from mass spectrometric analysis of rat [24], human [25] and mouse [26] GSTs led to the conclusion that only rGSTA4 has an N-terminal methionine residue. Recombinant proteins obtained from *E. coli* might not have authentic N-termini and the effect of an additional N-terminal residue on the structure and function of GSTs has not been fully explored.

Here we describe an *E. coli* expression system that produces recombinant proteins that are enriched in the N-terminal processed form. We chose cGSTM1-1 [27] and rGSTT1-1 [12] as model proteins for this study, where c and r refer to chick and rat respectively. These two isoenzymes isolated from tissues carry an N-terminal valine residue. The expression of rGSTT1-1 in *E. coli*

Abbreviations used: GST, glutathione S-transferase (the GST subunits are designated according to the class-based subunit nomenclature proposed by Mannervik et al. [Biochem. J. (1992) 282, 305–306]; Met-AP, methionine aminopeptidase; cGSTM1-1(Met-AP) and rGSTT1-1(Met-AP), GSTs co-expressed with extrinsic Met-AP gene, where c and r refer to chick and rat respectively; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; LC/MS, liquid chromatography/MS; NBC, 4-nitrobenzyl chloride.

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has not been described. We also report a rapid method for the purification of the recombinant rGSTT1-1 and the enzymic activities of this isoenzyme.

MATERIALS AND METHODS

Materials

Restriction enzymes and DNA-modification enzymes were obtained from New England Biolabs (Beverly, MA, U.S.A.). *Taq* I polymerase was purchased from Promega Corp. (Madison, WI, U.S.A.). The oligonucleotide-directed *in vitro* mutagenesis kit was purchased from Amersham (Little Chalfont, Bucks., U.K.). The Sequenase kit was obtained from US Biochemical (Cleveland, OH, U.S.A.). Adenosine 5'-[α -³⁵S]thio]triphosphate for sequencing was purchased from New England Nuclear (Wilmington, DE, U.S.A.). Glutathione, epoxy-activated Sepharose 6B and substrates used in enzymic assays were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The glutathione-Sepharose affinity column was coupled as described previously [28]. Preparative grade Superose 12 and Q-Sepharose Fast Flow were purchased from Pharmacia Biotech (Uppsala, Sweden). Macro-Prep ceramic hydroxyapatite was obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.). The Vydac C₁₈ reverse-phase columns were purchased from H-P Separations Group (Hesperia, CA, U.S.A.). Chemicals for protein sequencing were obtained from Applied Biosystems (Foster City, CA, U.S.A.). All other chemicals used were of reagent grade or better.

Construction and expression of cGSTM1-1 and rGSTT1-1 in *E. coli*

Oligonucleotides and primers used in molecular cloning and mutagenesis are listed in Table 1. The Bluescript phagemid (pGCL201) containing the coding region of the cGSTM1 subunit has been described previously [29]. A unique *Nde*I restriction endonuclease site was introduced into pGCL201 at the starting codon of the cGSTM1 gene by site-directed mutagenesis [30] with primer 1. The entire coding region of cGSTM1 was sequenced and the *Nde*I-*Sal*I fragment (799 bp) was inserted into the pBAce expression vector [31] to generate the pBAce-M1 plasmid (Figure 1A).

Two partial clones, together containing the entire coding sequence of rGSTT1 [32], were generously provided by Dr. Sally Pemble (University College London, London, U.K.). Plasmid pGST5L11 has a 260 bp insert and encodes the N-terminal 86 residues of rGSTT1. It was used as a template and amplified with primers 2 and 3 by PCR. The PCR product obtained was flanked by *Nde*I and *Pst*I restriction sites. Plasmid pGST5A27 has a

839 bp insert with a sequence starting at nt 91 of the rGSTT1 cDNA [32]. Primers 4 and 5 were used as PCR primers with pGST5A27 as template for generating a DNA fragment with a *Pst*I site and a *Sal*I site on the end. After digestion with appropriate restriction enzymes, the PCR products were joined at the *Pst*I site and inserted into the pBAce vector to generate the pBAce-T1 plasmid (Figure 1B). The pBAce-M1 and pBAce-T1 plasmids were transformed into *E. coli* TG1 cells for protein expression, as outlined by Liu et al. [33].

Cloning of *E. coli* Met-AP

The Met-AP gene was amplified by PCR with *E. coli* strain TG1 genomic DNA as a template. Primers 6 and 7 were constructed in accordance with the first and last 20 nucleotides of the reported *E. coli* Met-AP gene sequence [2] and used as primers in the reaction. The amplification product contained the coding region as well as the promoter region of the Met-AP gene. This fragment was digested with *Ava*I to remove part of the 5' region and inserted into a Bluescript vector. Before ligation the Bluescript vector was first treated with *Eco*RI and the cohesive ends were filled in with Klenow enzyme. The vector was then digested with *Ava*I and ligated with the pretreated PCR product. The resulting plasmid was designated pBS-MP (Figure 1C).

Construction of vectors for co-expression of GST and Met-AP

The phagemid pBS-MP was used as a template in a PCR amplification. Primers 8 and 9 were used to introduce a *Nde*I site and a *Sal*I site at the ATG start codon and the 3' non-coding region of the Met-AP gene respectively. The PCR product was digested with these two enzymes, then inserted into a pBAce vector (pBAce-MP). The *Kpn*I-*Xba*I fragment was excised from the pBAce-MP plasmid, and introduced into the *Kpn*I site of the pBAce-M1 vector with a *Xba*I-*Kpn*I adapter (5'-CATGGTAC-3'). The cGSTM1 and Met-AP genes were under the control of separate *phoA* promoters. The resulting vector was designated pBAce-MP-M1 (Figure 1D) and was transformed into TG1 cells for protein expression as outlined above.

Alternatively, the pBS-MP phagemid was first digested with *Xba*I endonuclease. The *Xba*I-*Kpn*I fragment from the pBAce-M1 vector was then ligated into the pBS-MP phagemid with a *Xba*I-*Kpn*I adapter to generate the pBS-M1-MP (Figure 1E) plasmid. The pBS-T1-MP (Figure 1F) plasmid was constructed by digesting the pBS-MP phagemid with *Hind*III and *Xba*I endonucleases. The *Eco*RI-*Xba*I fragment from the pBAce-T1 vector was then ligated into the pBS-MP phagemid with a *Hind*III-*Bam*HI-*Eco*RI linker (5'-AAGCTTATGGATCCAGGAATTC-3'). With these constructions, the expression of the Met-AP gene is under the control of its natural promoter, whereas that of the cGSTM1 or rGSTT1 gene is under the control of the *phoA* promoter. These plasmids were transformed into TG1 cells and the proteins were overexpressed as outlined above.

Purification of cGSTM1

All purification steps were performed at 4 °C. Harvested cells were suspended in buffer A [10 mM Tris/HCl (pH 8.0)/1 mM EDTA/0.2 mM dithiothreitol/0.5 mM PMSF] with 20% (w/v) sucrose at a concentration of 25 ml/l of cell culture. Cells were lysed by passing through a microfluidizer (Model EmulsiFlex-5C; Avestin, Ottawa, ON, Canada) and debris was removed by ultracentrifugation in a Beckman 70 Ti rotor for 1.5 h at 257000 g. The expressed proteins were loaded on a GSH affinity column that had been pre-equilibrated with buffer A. After being

Table 1 Primers used in molecular cloning

Primer	Sequence
1	5'-CAACGTGACCACUCATATGATTCGGATCCCCGGG-3'
2	5'-TGCAGGTCCCATATGGTGCTGGAGCTCTATCTG-3'
3	5'-CGGGCCTGCAGGTCTTGGGGTACCAGTGGTCA-3'
4	5'-ACGCCCAAGACCTGCAGGCCCGTCTCGTGTGG-3'
5	5'-GTGTAATGCGAGTCGACTGGCTGGTGCAGG-3'
6	5'-GATCGGAAGTCCGGCGCGCT-3'
7	5'-GCTTTTATCCACCGACGGT-3'
8	5'-GGGCGGCGGCCATATGGGGCTGGAGCTGTACCTGG-3'
9	5'-GGCCGTCGACAGGTGTAGAGCTGGAGATCAC-3'

Figure 1 Construction of expression plasmids for cGSTM1-1 and rGSTT1-1

The cGSTM1 (**A**) and rGSTT1 (**B**) genes were expressed under the control of the *phoA* promoter; (**C**) the Met-AP gene was expressed under the control of its natural promoter; (**D**) the cGSTM1 and the Met-AP genes were expressed under the control of separate *phoA* promoters; the cGSTM1 (**E**) and rGSTT1 (**F**) genes were expressed under the control of the *phoA* promoter, whereas the Met-AP gene was expressed under the control of its natural promoter.

washed with buffer A containing 0.2 M NaCl, the cGSTM1 subunits were eluted off the column with buffer B [10 mM 3-(cyclohexylamino)propane-1-sulphonic acid (pH 11.0)/1 mM EDTA/0.2 mM dithiothreitol/500 mM NaCl/5 mM GSH]. Proteins after affinity column chromatography were desalted immediately by gel-permeation chromatography on a preparative Superose 12 column that was equilibrated with 150 mM potassium phosphate, pH 7.5. The protein samples were stored at -70°C at a concentration of 1 mg/ml or more in the presence of 5 mM GSH.

Purification of rGSTT1

Purification of rGSTT1 subunits was performed at room temperature. Cells containing expressed rGSTT1-1 subunits were resuspended in buffer C [10 mM sodium phosphate (pH 7.0)/0.2 mM dithiothreitol/10% (v/v) glycerol]. Cells were lysed and the debris was removed by centrifugation as outlined above. The supernatant from 2 litres of cell cultures was loaded on a Q-Sepharose Fast Flow column (2.5 cm $\times$ 10 cm) pre-equilibrated in buffer C. The rGSTT1 subunits were recovered from the column flow through and loaded on a type I (20 μm) ceramic hydroxyapatite column (2.5 cm $\times$ 10 cm). After sample loading, the column was washed with 90 ml of buffer C, then eluted with a linear gradient of 0–35% buffer D [400 mM sodium phosphate (pH 7.0)/0.2 mM dithiothreitol/10% (v/v) glycerol] in 21 ml, followed by 35–80% buffer D in 270 ml. The rGSTT1-1 fractions

were located by enzymic assay with 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) as second substrate. Active fractions were eluted from the column at approx. 220 mM sodium phosphate [55% buffer D] and were dialysed against buffer C overnight. The dialysed sample was then loaded on the same hydroxyapatite column pre-equilibrated in buffer C. After sample loading, the column was washed with 60 ml of buffer C and eluted with a linear gradient of 0–100% buffer E (buffer C containing 1 M NaCl) in 300 ml. The rGSTT1 subunits were eluted from the column at 78% buffer E.

The purity of the recombinant GSTs was analysed with SDS/PAGE [34]. The molecular mass of the recombinant proteins were determined by electrospray MS as described by Yeh et al. [24]. The N-terminal sequences of the expressed proteins were confirmed and quantified by Edman degradation [35].

Liquid chromatography MS (LC/MS) analysis of recombinant proteins

The purified recombinant proteins were analysed on a Vydac (C_{18} ; 1 mm $\times$ 250 mm) reverse-phase column at a flow rate of 28 $\mu\text{l}/\text{min}$; the elution profile was monitored at 214 nm. A splitter was employed after the detector microflow cell, and approx. 7–8 μl of sample/min was introduced into the mass spectrometer. The column was equilibrated with 30% solvent 1 [0.08% (w/v) trifluoroacetic acid in water] and 70% solvent 2

[0.05 % (v/v) trifluoroacetic acid in 80 % (v/v) acetonitrile], then washed with the same buffer for 10 min after sample loading. The cGSTM1 and rGSTT1 subunits with and without the initiator methionine residue were separated with a linear gradient of 30–60 % solvent B in 5 min, then 60–70 % solvent B in 30 min.

Electrospray MS was performed on a Quattro-BIO-Q mass spectrometer (Micromass, Altrincham, Greater Manchester, U.K.) as previously described [24]. For determination of the molecular mass of GSTs, the multiply charged ion peaks from myoglobin (16951 Da) were used as an external reference for mass-scale calibration. Data were summed in accordance with the total ion current profile and processed by the MaxEnt program [36].

Enzymic assay

Enzyme activities were determined at 25 °C for cGSTM1-1 as described previously [37,38]. The enzymic activities of rGSTT1-1 were determined at 37 °C to permit a direct comparison with published data [12]. The rates of all the enzymic reactions were obtained by subtracting the non-enzymic rate from the observed reaction rates. The absorption coefficient of the recombinant protein was determined by using the protocol of Gill and von Hippel [39]. Protein solutions of cGSTM1-1, cGSTM1-1(Met-AP), rGSTT1-1 and rGSTT1-1(Met-AP) with one A_{280} unit corresponded to concentrations of 0.56, 0.50, 0.65 and 0.74 mg/ml respectively. rGSTM1-1(Met-AP) and rGSTT1-1(Met-AP) were recombinant GSTs co-expressed with extrinsic Met-AP gene.

RESULTS AND DISCUSSION

We have previously reported the expression of rGSTM1-1 [33], cGSTA1-1 and cGSTA2-2 [40] in *E. coli* under the control of a *phoA* promoter. These GSTs have a proline, an alanine and a serine residue respectively at the N-terminus. The radii of gyration of the side chains from these residues are relatively small [41] and the initiator methionine residues can be removed efficiently from the expressed proteins by the intrinsic Met-AP in the host cells. A literature search revealed the presence of a more bulky N-terminal valine residue on the cGSTM1 [27] and the rGSTT1 [12] subunits. We therefore put the cGSTM1 and the rGSTT1 cDNA species under the control of the *phoA* promoter and examined whether the N-terminus of the recombinant proteins could be efficiently processed.

Expression and purification of cGSTM1

The cGSTM1 cDNA was inserted into a pBAce vector (Figure 1A) for protein expression. The crude cell lysate was analysed on a denaturing gel and showed clearly that cGSTM1 was over-expressed (Figure 2, lane 2). The cGSTM1 subunits exhibited a strong affinity for the glutathione affinity matrix, resulting in low protein recovery under normal elution conditions [28]. Quantitative recovery of the protein could be obtained by including 5 mM *S*-hexylglutathione in the elution buffer. However, the *S*-hexylglutathione could not be effectively removed from the enzyme by gel-permeation chromatography or dialysis. LC/MS analysis of samples thus prepared revealed the presence of approx. 0.75 mol of *S*-hexylglutathione per mol of GST subunit (results not shown).

We modified the purification procedure by eluting the protein from the glutathione-affinity column at pH 11 in the presence of 0.5 M NaCl and 5 mM GSH. The proteins were desalted immediately by gel permeation; 30–35 mg of proteins could be obtained from 1 litre of cell culture. LC/MS analysis indicates

Figure 2 Denaturing PAGE of recombinant GSTs

From left to right the first six lanes are: cell lysates from TG1 cells, and from TG1 cells harbouring pBAce-M1, pBAce-MP-M1, pBS-M1-MP, pBAceT1 and pBS-T1-MP plasmids. The remaining three lanes are rGSTT1 samples before and after the first hydroxyapatite (HA) column and after the second hydroxyapatite column respectively. Proteins were revealed by staining with Coomassie Brilliant Blue.

the presence of two proteins with molecular masses of 25761 ± 2 and 25892 ± 2 Da at a 1.5:8.5 ratio in our preparation (Figure 3A). The values agree with that of cGSTM1 predicted from cDNA sequencing (25762 Da) and subunits retaining the initiator methionine (25893 Da) respectively. Edman degradation confirmed the presence of the initiator methionine residue on the protein from the major peak. This residue was completely removed from the mature cGSTM1 isolated from chick livers [27].

Expression and purification of rGSTT1

Expression of recombinant rGSTT1-1 in *E. coli* has not been reported. The complete coding sequence of rGSTT1 was reconstructed from two partial clones and inserted into the pBAce vector for expression (Figure 1B). The level of rGSTT1 expression is comparable to that of cGSTM1 as analysed with SDS/PAGE (Figure 2, lane 5).

The rGSTT1 subunits were partly isolated from cellular proteins by passing the lysates through an anion exchanger (Q-Sepharose Fast Flow) at pH 7.0. The unbound fraction (Figure 2, lane 7) containing rGSTT1 was separated on a hydroxyapatite column with a phosphate gradient (Figure 4A). Fractions with EPNP-conjugating activity (Figure 2, lane 8) were collected and rechromatographed on the same hydroxyapatite column with an NaCl gradient (Figure 4B). The purified rGSTT1 was more than 95 % pure (Figure 2, lane 9). LC/MS analysis (Figure 3E) indicated that the sample has two components with molecular masses of 27478 and 27337 Da in a 9:1 ratio. Edman degradation confirmed that these were rGSTT1 subunits with and without the N-terminal methionine residue respectively.

The EPNP-conjugating activity of the enzyme during purification diminished rapidly at 4 °C. However, the activity of the

Figure 3 HPLC profiles of recombinant GSTs

Purified recombinant GSTs from various expression plasmids were analysed on a C_{18} reverse-phase column. **(A)** cGSTM1 from the pBAce-M1 plasmid; **(B)** cGSTM1 from the pBAce-MP-M1 plasmid; **(C)** cGSTM1 from the pBS-M1-MP plasmid; **(D)** cGSTM1 from the pBS-M1-MP plasmid, grown with 0.25 mM phosphate in the culture medium; **(E)** rGSTT1 from the pBAce-T1 plasmid; **(F)** rGSTT1 from the pBS-T1-MP plasmid.

Figure 4 Elution profiles of the recombinant rGSTT1 on a hydroxyapatite column

(A) Cell lysates from TG1 cells harbouring pBAce-T1 plasmids were loaded on a Q-Sepharose Fast Flow column and the flow-through fraction was further separated on a hydroxyapatite column (2.5 cm $\times$ 10 cm) with a phosphate gradient. **(B)** Fractions with EPNP activity were collected as indicated and reloaded on the same column, then eluted with a NaCl gradient.

crude lysate was stable at room temperature. Therefore all purification processes were performed at room temperature or at 25 °C. Sample eluted from the second hydroxyapatite column was frozen immediately with liquid nitrogen and stored at -70 °C. We routinely obtained 5–6 mg of purified rGSTT1 from 1 litre of cell culture. Our purification process involved only three columns and the whole task could be accomplished within 30 h. It was simpler and faster than those reported previously by Meyer et al. [12,42] and Hiratsuka et al. [43].

Co-expression of GST with Met-AP

Proteins with a penultimate valine residue, such as cGSTM1 and rGSTT1 in this study, are not ideal substrates for Met-AP. Furthermore these proteins are overexpressed, becoming an added burden to the intrinsic Met-APs of the host cells. Hirel et al. [8] proposed that the removal of the initiator methionine residue is a co-translational process and the number of Met-AP molecules per ribosome is limited. To increase the cellular Met-AP concentration, we inserted a Met-AP cDNA into the pBAce-M1 vector under the control of a separate *phoA* promoter (Figure 1D) and co-expressed it with the cGSTM1 gene. With this construct, the recombinant protein expressed was predominantly Met-AP (Figure 2, lane 3). After affinity column purification, only 2–3 mg of recombinant cGSTM1 was obtained from 1 litre of cell culture. The purified cGSTM1 was analysed by LC/MS; the result is presented in Figure 3(B). Approx. 60 % of the recombinant proteins had their initiator methionine residue removed.

Shen et al. [35,44] have reported the co-expression of human haemoglobin with Met-AP under separate *tac* promoters. The amount of Met-AP expressed is substantially higher than that of the haemoglobin subunits combined [44]. In addition, synthetic human α - and β -globin cDNA species with codon usage optimized for *E. coli* expression have been used. Apparently, *E. coli* preferentially expressed the bacterial Met-AP instead of eukaryotic genes.

In view of the low expression level and incomplete N-terminal processing of the cGSTM1, the overexpressed Met-AP might not be active or participate in the N-terminal processing of the recombinant GST. In addition, the recombinant Met-AP exists predominantly (more than 95 %) without its initiator methionine residue (results not shown). A large proportion of the intrinsic Met-AP probably engaged in processing the recombinant Met-AP instead of the GST subunits. We therefore put the cGSTM1 or rGSTT1 gene into the pBS-MP plasmid for protein expression (Figures 1E and 1F). With these constructs, the Met-AP gene was under the control of its natural promoter, constitutively expressed and accumulated in the host cells. Biosynthesis of recombinant GSTs was delayed until the phosphate had been depleted from the growth medium. We rationalize that the extra Met-AP pre-existing in the host cells could facilitate the removal of the initiator methionine residue without competing with the GSTs for the cellular translation and N-terminal processing machineries.

The protein expression patterns of cells hosting pBS-MP-M1 and pBS-MP-T1 are presented in Figure 2 (lanes 4 and 6 respectively). The expression of Met-AP was drastically decreased. The purified cGSTM1 and rGSTT1 were analysed with LC/MS; the chromatograms are presented in Figures 3(C) and 3(F). Approx. 60 % and 70 % of the initiator methionine residues have been removed from the recombinant cGSTM1 and rGSTT1 respectively. With these constructs, we could obtain 20 and 5 mg of purified cGSTM1 and rGSTT1 respectively from 1 litre of cell culture.

Under normal expression conditions, the growth medium contained 0.1 mM phosphate. We increased the concentration of phosphate to 0.25 mM to delay the expression of the GST genes: in this way we could obtain 85 % cleavage of the initiator methionine residue from cGSTM1 (Figure 3D). However, cleavage from rGSTT1 was not increased by increasing the phosphate concentration in the medium. Instead, the amount of initiator methionine residue cleavage from rGSTT1 was decreased to 65 % when the phosphate concentration in the medium was increased to 0.6 mM.

Wang et al. [45] have reported that the initiator methionine on recombinant apolipoprotein C-II can be removed by incubation with purified Met-AP. Successful removal of initiator methionine residues from folded proteins has also been reported for interleukin 2 and ricin A [2]. Therefore stoichiometric amounts of cells containing the pBAce-M1 or pBAce-T1 plasmid were mixed with samples containing pBS-MP or pBAce-MP plasmid. The cells were lysed in phosphate buffer, pH 7.5, containing 0.2 mM CoCl₂ so that the recombinant Met-AP could interact with the GSTs. After 3 h of incubation at 37 °C, the cGSTM1 or rGSTT1 subunits were separated with SDS/PAGE and transferred to PVDF membranes for N-terminal sequencing analysis. We could not detect any additional excision of initiator methionine residues on either GST by this incubation process *in vitro*. Our results support the notion [5,41] that excision of the initiator methionine residue is a co-translational process.

Characterization of recombinant GSTs

The substrate specificities of cGSTM1 and rGSTT1 expressed in the presence or absence of extrinsic Met-AP gene are summarized in Table 2. Data from Meyer et al. [12] and Hiratsuka et al. [43] on the activity of rGSTT1 are also included for comparison. Except for the peroxidase activity, the specific activities of the cGSTM1-1 isoenzymes, with or without the initiator methionine, are quite similar (Table 2) [46]. The cGSTM1-1(Met-AP) sample, containing approx. 14 % initiator methionine residue, was 20 % lower in the cumene hydroperoxide reduction activity. However, owing to the low peroxidase activity of cGSTM1-1, the observed discrepancy might have been the consequence of experimental error.

We further determined the kinetic properties of cGSTM1-1(Met-AP). The K_m values for GSH, CDNB and EPNP were 0.059, 1.39 and 0.67 mM respectively. The results are nearly identical with those obtained for cGSTM1-1 [46]. The k_{cat} values determined with CDNB and EPNP as second substrates were 24.0 ± 0.9 and 5.65 ± 0.42 s⁻¹. Again, the results obtained are similar to those for the cGSTM1-1 sample (30.9 and 4.33 s⁻¹ respectively [46]). The calculated k_{cat}/K_m values for cGSTM1-1 and cGSTM1-1(Met-AP) with these two substrates are within 10 % of each other. We conclude that the initiator methionine residue does not affect the CDNB-conjugating and epoxidase activities of cGSTM1-1.

The enzymic activities of rGSTT1-1 and rGSTT1-1(Met-AP) are listed in Table 2. Of the seven substrates tested, we could detect activities only towards EPNP, 4-nitrobenzyl chloride (NBC) and cumene hydroperoxide. The results obtained for the rGSTT1-1 and rGSTT1-1(Met-AP) samples are within 10 % of each other. The initiator methionine residue therefore has minimal effect on the activity of the enzyme.

Our conclusion is in general agreement with that of Lai et al. [47]. They expressed a variant of rGSTM1-1 lacking the N-terminal proline residue. This enzyme has similar physical and kinetic properties to its counterpart purified from rat livers. The N-terminal domain I of GSTs has been considered to be the

Table 2 Substrate specificities of cGSTM1-1 and rGSTT1-1Results are means $\pm$ S.E.M. for duplicates of at least three experiments. —, not determined; n.d., not detectable.

Substrate	Specific activity ($\mu\text{mol}/\text{min}$ per mg)					
	cGSTM1-1*	cGSTM1-1(Met-AP)	rGSTT1-1†	rGSTT1-1(Met-AP)†	rGSTT1-1‡	rGSTT1-1§
1-Chloro-2,4-dinitrobenzene	23.6 $\pm$ 0.5	22.1 $\pm$ 0.4	n.d.	n.d.	< 0.5	n.d.
1,2-Dichloro-4-nitrobenzene	n.d.	n.d.	n.d.	n.d.	—	—
Ethacrynic acid	0.29 $\pm$ 0.01	0.30 $\pm$ 0.02	n.d.	n.d.	—	—
EPNP	4.3 $\pm$ 0.08	4.1 $\pm$ 0.1	140.6 $\pm$ 3.2	152.9 $\pm$ 3.0	180	35.2
Cumene hydroperoxide	0.42 $\pm$ 0.02	0.34 $\pm$ 0.05	15.8 $\pm$ 1.6	15.9 $\pm$ 1.4	41	24.7
NBC	—	—	76.7 $\pm$ 2.2	82.6 $\pm$ 4.4	86	7.8
1-Menaphthyl sulphate	n.d.	n.d.	n.d.	n.d.	—	n.d.

* Data from [46].

† Data obtained at 37 °C reaction temperature.

‡ Data from [12].

§ Data from [43].

glutathione-binding domain. However, the N-terminus of GSTs of classes Mu and Theta are too far from the reaction centre to be involved in the enzymic mechanism [16,21,46,48].

The isolation from rat liver of a GST isoenzyme with high epoxidase activity was reported by Fjellstedt et al. [49] and Meyer et al. [42]. In these reports, the EPNP-conjugating activity of the isoenzyme performed at 25 °C was less than 30 $\mu\text{mol}/\text{min}$ per mg. The purification process was later simplified by using an Orange A column [12]; the rGSTT1-1 thus isolated has an EPNP-conjugating activity of 180 $\mu\text{mol}/\text{min}$ per mg. It should be noted that the higher epoxidase activity was obtained at 37 °C in the latter report.

Recently, Hiratsuka et al. [43] reported a strategy of isolating class Theta GSTs from rat liver cytosol. However, the specific activities of the purified rGSTT1-1 towards EPNP, NBC and cumene hydroperoxide are 1/5, 1/11 and 1/1.6 of those reported by Meyer et al. [12]. The peroxidase reaction was performed at 37 °C, whereas the EPNP and NBC reactions were performed at 25 °C with the method of Habig et al. [37].

The enzymic assays of our recombinant rGSTT1-1 were performed at 37 °C for direct comparison with Meyer's results [12]. We obtained specific activities of 152.9 $\pm$ 3.0 and 82.6 $\pm$ 4.4 $\mu\text{mol}/\text{min}$ per mg of protein towards EPNP and NBC respectively (Table 2). The results are only 15% and 4% lower than that reported by Meyer et al. [12]. We have also determined the specific activity for EPNP and NBC at 25 °C and obtained values of 89.7 $\pm$ 3.7 and 41.9 $\pm$ 4.0 $\mu\text{mol}/\text{min}$ per mg of protein respectively. The results are 2–5-fold those reported by Hiratsuka et al. [43]. The peroxidase activity (15.9 $\pm$ 1.4 $\mu\text{mol}/\text{min}$ per mg of protein) of our recombinant protein is substantially lower than that reported; the reasons for this remain to be investigated.

rGSTT1-1 is a rather labile enzyme. We lost 90% of the enzymic activity within 5 h of cell lysis when purification was performed at 4 °C. The enzyme in the cell lysate can maintain its activity at room temperature for 72 h. However, precipitation of the purified protein does occur at room temperature after 5 days. Apparently, the low activity observed by Hiratsuka et al. [43] was a combination of low reaction temperature and the presence of inactive enzymes.

In conclusion, we have reported here an expression system for the improved production of N-terminally processed recombinant proteins. We successfully expressed and purified rGSTT1-1 from *E. coli*. The enzymic activities of the recombinant rGSTT1-1 are comparable to those for the enzyme isolated from rat livers. The

initiator methionine residue has minimal effect on the enzymic activities of either cGSTM1-1 or rGSTT1-1.

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