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Short communication

Liquid chromatography–electrospray mass spectrometry study of cysteine-10 *S*-glutathiolation in recombinant glutathione *S*-transferase of *Ochrobactrum anthropi*

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Abstract

Glutathione *S*-transferase of *Ochrobactrum anthropi* (OaGST), a bacterium isolated from soils contaminated by xenobiotic pollutants, was recently purified, cloned and characterised in our laboratories. The recombinant OaGST (rOaGST), highly expressed in *Escherichia coli*, when purified by glutathione-affinity chromatography and then analysed by electrospray ionisation mass spectrometry (ESI-MS), has evidenced a disulphide bond with glutathione (*S*-glutathiolation), which was removable by reduction with 2-mercaptoethanol. Enzymatic digestion of rOaGST with endoproteinase Glu-C, followed by liquid chromatography (LC)–ESI-MS analyses of the peptide mixtures under both reducing and not reducing conditions, have shown that glutathione was covalently bound to the Cys10 residue of rOaGST. Furthermore, LC–ESI-MS analyses of overexpressed rOaGST in *Escherichia coli* crude extracts, with and without incubation with glutathione, have not shown any *S*-glutathiolation of the recombinant enzyme.

Keywords: *Ochrobactrum anthropi*; Cysteine; Glutathione *S*-transferase

1. Introduction

Glutathione *S*-transferases (GSTs; EC 2.5.1.18) are a family of multi-functional dimeric enzymes that catalyse the conjugation of the tripeptide glutathione (GSH; Glu–Cys–Gly) to a large variety of electrophilic compounds of both endobiotic and xenobiotic origins. GSTs are involved in the metabolisation of naturally occurring toxins and reactive oxygen species and in the resistance to cancer chemotherapy agents, insecticides, herbicides and microbial antibiotics. These enzymes have two active sites per dimer and each active site consists of two ligand binding regions: the GSH binding site (G-site) is highly specific for GSH, whereas the binding site for the electrophilic substrate (H-site) is able to react with a broad range of toxic compounds (see Refs. [1–6] for reviews).

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Protein *S*-glutathiolation, that is the formation of a disulphide bond with GSH on protein Cys residues, has been observed in both eukaryotic [7–12] and prokaryotic [13] GSTs. Since the Cys residues seem not to be involved in the catalytic activity of eukaryotic GSTs [1,4] and of the bacterium *Proteus mirabilis* [14] GST (although the contrary has been supposed for *Escherichia coli* [15]), it was suggested that mixed disulphides may play a role in the regulation of the enzymatic activity, as observed for a large variety of other proteins [16,17]. However, *S*-glutathiolation in *Schistosoma* spp. GSTs [7,18] was attributed to the conventional GSTs purification system by GSH-affinity chromatography, using GSH as eluting agent [19]. On the other hand, a mixed disulphide was recently described in a rat GST purified without the use of GSH in the elution buffer [12].

In the last decade, mass spectrometry, as a result of its high speed and flexibility, has become a very powerful technique for protein molecular mass determination and for the detection of post-translational modifications in recombinant and wild-type proteins [20–22]. In GSTs studies, mass spectrometry analysis has often been used as a tool for the characterisation of eukaryotic GST isoenzymes and of their distribution in different tissues, for peptide mapping and sequencing.

The GST of *Ochrobactrum anthropi* (OaGST; Fig. 1), a bacterium isolated from polluted soils [23] and modulated in vivo by xenobiotics [24], was purified and cloned in our laboratories [25,26]. In order to better understand the detoxification mechanisms of *Ochrobactrum anthropi*, OaGST and other enzymes potentially involved in the degradation of xenobiotics are currently under investigation [27].

Here is described the electrospray ionisation mass spectrometry (ESI-MS) analysis of *Ochrobactrum anthropi* recombinant GST (rOaGST) that has evidenced a *S*-glutathiolation of the enzyme. Further-

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   1      ATCGAAACTGATGGGAGATTTTT   ATG AAG CTT TAC TAC AAG GTC GGC GCT TGT TCG CTC    59
   1                                Met Lys Leu Tyr Tyr Lys Val Gly Ala Cys Ser Leu    12

  60  GCA CCC CAC ATC ATT CTG AGC GAG GCG GGC CTG CCT TAT GAG CTG GAG GCC GTG   113
  13  Ala Pro His Ile Ile Leu Ser Glu Ala Gly Leu Pro Tyr Glu Leu Glu Ala Val    30

 114  GAT CTC AAG GCC AAG AAG ACA GCG GAC GGT GGC GAT TAT TTC GCA GTC AAT CCG   167
  31  Asp Leu Lys Ala Lys Lys Thr Ala Asp Gly Gly Asp Tyr Phe Ala Val Asn Pro    48

 168  CGC GGT GCG GTC CCG GCG CTG GAA GTG AAG CCC GGC ACT GTC ATC ACG CAG AAT   221
  49  Arg Gly Ala Val Pro Ala Leu Glu Val Lys Pro Gly Thr Val Ile Thr Gln Asn    66

 222  GCG GCA ATT CTC CAA TAT ATC GGT GAT CAT TCC GAT GTT GCA GCA TTC AAG CCC   275
  67  Ala Ala Ile Leu Gln Tyr Ile Gly Asp His Ser Asp Val Ala Ala Phe Lys Pro    84

 276  GCC TAT GGT TCA ATC GAA CGC GCA CGC CTG CAG GAA GCG TTG GGC TTC TGT TCG   329
  85  Ala Tyr Gly Ser Ile Glu Arg Ala Arg Leu Gln Glu Ala Leu Gly Phe Cys Ser   102

 330  GAT TTG CAT GCG GCC TTT AGC GGC CTG TTC GCG CCC AAC CTG AGC GAG GAA GCG   383
 103  Asp Leu His Ala Ala Phe Ser Gly Leu Phe Ala Pro Asn Leu Ser Glu Glu Ala   120

 384  AGG GCT GGC GTC ATC GCC AAC ATC AAT CGT CGT CTG GGT CAG CTC GAA GCC ATG   437
 121  Arg Ala Gly Val Ile Ala Asn Ile Asn Arg Arg Leu Gly Gln Leu Glu Ala Met   138

 438  CTG TCG GAC AAG AAC GCC TAC TGG CTT GGC GAT GAC TTC ACT CAA CCA GAT GCC   491
 139  Leu Ser Asp Lys Asn Ala Tyr Trp Leu Gly Asp Asp Phe Thr Gln Pro Asp Ala   156

 492  TAT GCG TCG GTG ATC ATC GGT TGG GGC GTT GGT CAA AAG CTC GAT TTG AGC GCC   545
 157  Tyr Ala Ser Val Ile Ile Gly Trp Gly Val Gly Gln Lys Leu Asp Leu Ser Ala   174

 546  TAT CCC AAG GCG CTG AAA CTG CGC GAA CGT GTG CTG GCC CGC CCG AAC GTG CAG   599
 175  Tyr Pro Lys Ala Leu Lys Leu Arg Glu Arg Val Leu Ala Arg Pro Asn Val Gln   192

 600  AAG GCA TTC AAG GAA GAA GGC CTG AAC TAA     AAATACAGCCGGAGGTCCAAACCTCCGGCTG    660
 193  Lys Ala Phe Lys Glu Glu Gly Leu Asn stop                                   201
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Fig. 1. Nucleotidic and deduced amino acidic sequence of rOaGST (accession number P81065). The average M_r corresponding to the amino acidic sequence is 21 738.9 u. The two possible sites of *S*-glutathiolation (Cys10 and Cys101) are pointed out.

more, liquid chromatography–ESI-MS (LC–ESI-MS) analyses were performed both on digested purified rOaGST, in order to determine the *S*-glutathiolation site, and on overexpressed rOaGST in *Escherichia coli* crude extracts, to verify whether the *S*-glutathiolation was present also in the non-purified enzyme.

2. Materials and methods

2.1. Chemicals

Glutathione (GSH), 2-mercaptoethanol (2-ME) and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Sigma (St. Louis, MO, USA), Bio-Rad Laboratories (Richmond, CA, USA) and Eurobio (Les Ulis, France), respectively. Endoproteinase Glu-C, excision grade (EC. 3.4.21.19), was obtained from Calbiochem (La Jolla, CA, USA). Acetonitrile (HPLC grade) and other chemicals (analytical grade) were purchased from Carlo Erba Reagenti (Milano, Italy). Sterile deionised water was purchased from Laboratori Diaco Biomedicali (Trieste, Italy). Phosphate-buffered saline (PBS) and Luria Bertoni medium (LB) were prepared as described elsewhere [28]. Components of LB were purchased from Difco (Detroit, MI, USA).

2.2. rOaGST expression and purification

Escherichia coli (*E. coli*) XL1-Blue cells, transformed with pT-OaGST [26], were grown overnight at 37 °C in LB medium, diluted 1:10 and grown in fresh LB medium until the A_{600} reached 0.4. To induce gene transcription, IPTG was added to a final concentration of 1 m*M* and the incubation was prolonged for another 5 h. Cells were harvested by centrifugation (10 000 *g* for 15 min), resuspended in 10 m*M* potassium phosphate buffer at pH 7, and disrupted by cold sonication. The particulate material was removed by centrifugation at 100 000 *g*, and the supernatant was loaded onto a glutathione–sepharose 4B column (Amersham–Pharmacia Biotech, Uppsala, Sweden). The column was washed with PBS, and the overexpressed protein, which was bound to the affinity column, was eluted with 10 m*M* GSH in 50 m*M* Tris–HCl buffer at pH 8. The rOaGST fractions were then dialysed with deionised water and concentrated using Centriprep-10 concentrators (Amicon, Beverly, MA, USA). Protein concentration was determined by the bicinchoninic acid assay (BCA) (Pierce, Rockford, IL, USA) and protein purity was tested by polyacrylamide gel electrophoresis (12.5% polyacrylamide gel) in the presence of sodium dodecylsulfate (SDS–PAGE) with silver staining detection (Ref. [28] and Refs. therein).

2.3. rOaGST digestion

Thirty μl of 20 m*M* ammonium acetate buffer at pH 4.0 containing 2.5 μg/μl of rOaGST and 100 ng/μl of endoproteinase Glu-C were incubated for 2 h at 37 °C in a 1.5-ml microfuge tube (Eppendorf, Hamburg, Germany). Two aliquots of 10 μl of the digestion mixture were diluted with 10 μl of water and then one of the two was reduced with 2% of 2-ME for 30 min at room temperature. Controls were constituted of the same components, without rOaGST, and were treated at the same conditions of the samples.

2.4. ESI-MS and LC–ESI-MS analyses

Analyses were performed using an API 365 triple quadrupole mass spectrometer (Sciex, Toronto, Canada) operated in positive ion mode. The mass spectrometer was previously calibrated with polypropylene glycol (PE Sciex, Foster City, CA, USA), setting the resolution at 0.7 ± 0.1 u. Horse heart myoglobin standard (Sigma) at the concentration of 1 mg/ml was used as reference standard for protein mass calibration. Instrument control and data acquisition were performed with a Macintosh G4/400 computer (Apple, Cupertino, CA, USA) using Masschrom 1.1.1 softwares (PE Sciex). LC analyses were performed using a Perkin-Elmer series 200 micro LC pump system (Norwalk, CT, USA) coupled to the mass spectrometer through a Turbo-IonSpray source (Sciex). Samples were manually injected by means of a model 7725i Rheodyne valve (Rohnert Park, CA, USA) equipped with a 20-μl sample loop. LC columns and chromatographic conditions are described below in Sections 2.4.2. and 2.4.3.

2.4.1. ESI-MS analyses of purified rOaGST

Purified proteins (1 μg/μl in water containing 0.1% formic acid) were infused at the flow-rate of 5 μl/min into an IonSpray source (Sciex) through a fused-silica capillary (1 m×75 μm I.D.; 150 μm O.D.; Polymicro Technologies, Phoenix, AZ, USA) using a model 11 infusion pump (Harvard Apparatus, South Natick, MA, USA). The nebulizer gas flow (air) and the curtain gas flow (N_2) were set at 2.1 and 2.4 l/min, respectively. The ionspray, orifice and ring voltages were set at +5000, +50 and +280 V, respectively. Mass spectra were acquired over the range m/z 300–2000 u using a step size of m/z 0.1 u and a dwell time of 0.250 ms (scan time 4.25 s). Each spectrum was a sum of 35 scans. Protein M_r were calculated using the BioSpec Reconstruct algorithm of the Biomultiview 1.3.1 software (PE Sciex).

2.4.2. LC–ESI-MS analysis of digested rOaGST

Analyses of the digested protein were performed using a Brownlee RP Aquapore OD-300 microbore column (100×1.0 mm, C_{18}, 7 μm, 300 Å) purchased from Perkin-Elmer. Twenty μl of digested protein (prepared as described in Section 2.3.) were

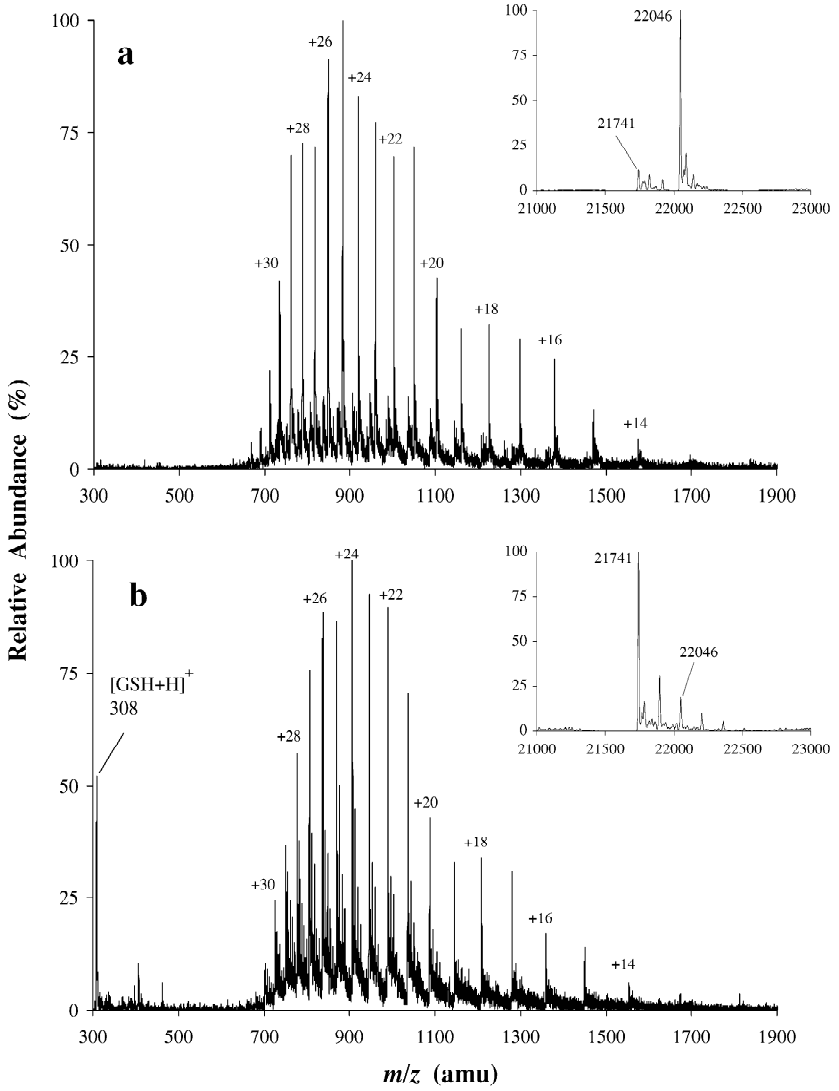

Fig. 2. Mass spectra of purified rOaGST (a) before and (b) after treatment with 2-ME.

injected and separations were carried out using a linear gradient of acetonitrile (A) in water (B), both with 0.5% of formic acid (from 10 to 60% of A in 60 min) at the flow-rate of 80 μl/min. The LC system was directly connected to the TurboIonSpray source. The nebulizer gas flow (air) and the curtain gas flow (N_2) were set at 2.8 and 2.4 l/min, respectively. The turboprobe was heated at 300 °C and the auxiliary gas flow (air) set at 3 l/min. The ionspray, orifice and ring potentials were set at +5000, +50 and +280 V, respectively. Mass spectra were acquired over the range m/z 200–3000 u using a step size of m/z 1.0 u and a dwell time of 0.540 ms (scan time 1.4 s). Data handling was performed with the Biomultiview 1.3.1 software. M_r greater than 3000 u were calculated with the Manual Reconstruction algorithm, using at least three peaks of the multicharged peptides.

2.4.3. LC–ESI-MS analysis of bacterial extracts

Analyses of bacterial extracts (obtained as described in Section 2.2.) were performed using a RP Supelcosil LC-318 column (50×4.6 mm, C_{18}, 5 μm, 300 Å) equipped with a Supelguard guard column (20×4.0 mm, C_{18}, 5 μm, 300 Å), both purchased from Supelco (Bellefonte, PA, USA). Twenty μl of bacterial extract at a total protein concentration of 10 mg/ml were injected and separations were carried out using a linear gradient of acetonitrile (A) in water (B), both with 0.5% of formic acid (25% A for 2 min, then to 50% of A in 60 min) at the flow-rate of 800 μl/min. The LC system was connected to the TurboIonSpray source after post-column splitting (1:10). The mass spectrometer parameters were the same as described in Section 2.4.2, and mass spectra were acquired over the range m/z 300–2000 u using a step size of m/z 1.0 u and a dwell time of 1.0 ms (scan time 1.7 s). Protein M_r were calculated using the BioSpec Reconstruct algorithm of the Biomultiview 1.3.1 software.

3. Results and discussion

3.1. ESI-MS analyses of purified rOaGST

rOaGST was overexpressed in *E. coli* and purified as described in Section 2.2, giving a single band at the apparent M_r of 24 000 u when analysed by SDS–PAGE [26]. Mass spectrometry was used to confirm the expected M_r of rOaGST and to investigate the occurrence of possible post-translational modifications. The reconstructed mass spectrum of rOaGST (Fig. 2a) has shown a M_r of 22 046 u, then exceeding the expected M_r (21 740 u) deduced from the gene sequence (Fig. 1) by 306 u, which might correspond to the *S*-glutathiolation of one Cys residue. As shown in Fig. 2b, the M_r of rOaGST shifted to the expected M_r when the enzyme was reduced with 2-ME for 30 min at room temperature (higher incubation temperatures were not used in order to avoid possible non-catalytic reactions due to heating). Moreover, the mass spectrum showed a peak at m/z 308 u corresponding to GSH protonated molecular ion (Fig. 2b). These data strongly evidenced the presence of a disulphide bond between GSH and rOaGST.

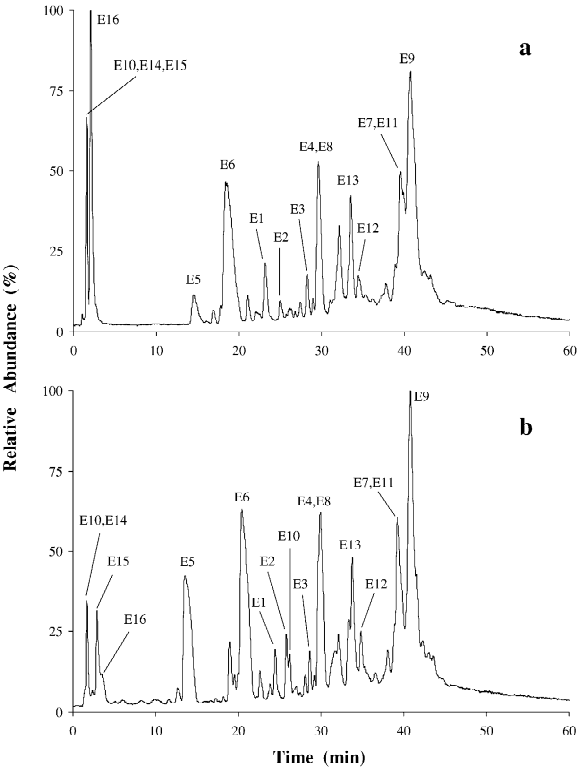

Fig. 3. Total ion chromatograms (a) before and (b) after treatment with 2-ME of rOaGST enzymatically digested with endoproteinase-Glu. Peaks are identified in Table 1.

3.2. LC–ESI-MS analyses of digested rOaGST

From the primary structure reported in Fig. 1, only two Cys residues of rOaGST (Cys10 and Cys101) might be the possible sites of the *S*-glutathiolation and to determine which of the two was involved in the disulphide bond with GSH, rOaGST was digested with endoproteinase Glu-C. This peptidase, in the presence of ammonium acetate at pH 4.0, specifically hydrolyses peptide bonds at the carboxylic side of glutamic acid residues [29]. The enzymatically derived peptides were directly analysed by LC–ESI-MS, with and without reduction with 2-ME (Fig. 3). All the identified peptides are reported in Table 1. The peptide localised at 1–20 in the rOaGST sequence, which is a Cys10 containing fragment (Fig. 1), gave rise to two different forms: peptide E1 corresponding to the glutathiolated form (Fig. 4a; m/z 2544 u), and peptide E2 corresponding to the non-glutathiolated form (Fig. 4b; m/z 2238 u). The extracted chromatograms of the most abundant ions, m/z 848 u and m/z 747 u for the glutathiolated and non-glutathiolated forms, respectively, have shown that treatment with 2-ME determined at the same time a doubling of peptide E2 area and a halving of peptide E1 area. After extracting the chromatograms of their most abundant ion, similar results were obtained for peptide E3 and E4 derived from the glutathiolated and non-glutathiolated forms of the 1–28 residues, respectively, even if these peptides were much less abundant than the corresponding 1–20 residues. As shown in Fig. 4, there is a difference in the number of multiple charged ions in the two peak spectra: four in the peptide E1 spectrum and three in the peptide E2 spectrum. As previously described [20,30], multiple charged ions of proteins and peptides are due to the basic groups protonated at low pH (e.g., Arg, His, Lys, N-terminal free amine group). The 1–20 residues contain two Lys, one His and the N-terminal amine group; therefore we may expect a maximum of four charges for this peptide. In our analytical conditions (ionisation potential, solvents used, etc.) we found only three charges, and, when glutathiolated, the 1–20 residues have shown a fourth charge that is compatible with the N-terminal amine group of GSH after *S*-glutathiolation. Furthermore, peptides containing Cys101 residue have not evidenced any *S*-glutathiolation. These data are in agreement with a previous study in which a mixed disulphide bond with GSH

Table 1
Peptides identified during the on-line LC–ESI-MS analyses of digested rOaGST (Fig. 3)

Peptide	Position	Sequence	Calculated mass[a] (u)	Observed mass[b] (u)	
				rOaGST digests (not reduced)	rOaGST digests (reduced)
E1	1–20	<MKLYYKVGAC*SLAPHIILSE>A	2543.06	2543	2543
E2	1–20	<MKLYYKVGACSLAPHIILSE>A	2236.73	2237	2237
E3	1–28	<MKLYYKVGAC*SLAPHIILSEAGLPYELE>A	3416.03	3416	3416
E4	1–28	<MKLYYKVGACSLAPHIILSEAGLPYELE>A	3109.70	3111	3111
E5	21–28	E<AGLPYELE>A	890.44	891	891
E6	29–56	E<AVDLKAKKTADGGDYFAVNPRGAVPALE>V	2874.25	2875	2875
E7	29–118	E<AVDLKAKKTADGGDYF . . . LHAAFSGLFAPLNSE>E	9434.66	9434	9435
E8	57–90	E<VKPGTVITQNAAILQ . . . HSDVAAFKPAYGSIE>R	3575.04	3576	3576
E9	57–183	E<VKPGTVITQNAAILQ . . . KLDLSAYPKALKLRE>R	13 739.60	13 742	13 741
E10	91–96	E<RARLQE>A	771.44	772	772
E11	91–183	E<RARLQEALGFCSDLH . . . KLDLSAYPKALKLRE>R	10 182.58	10 186	10 185
E12	97–136	E<ALGFCSDLHAAFSGLF . . . AGVIANINRRLGQLE>A	4229.79	4231	4231
E13	137–183	E<AMLSDKNAYWLGDDF . . . KLDLSAYPKALKLRE>R	5216.95	5219	5218
E14	184–197	E<RVLARPNVQKAFKE>E	1655.96	1656	1657
E15	184–198	E<RVLARPNVQKAFKEE>G	1785.08	1785	1785
E16	184–201	E<RVLARPNVQKAFKEEGLN>	2069.44	2070	2070

C* are *S*-glutathiolated cystein residues.
[a] Calculated from the amino acidic sequence.
[b] Obtained through the analysis.

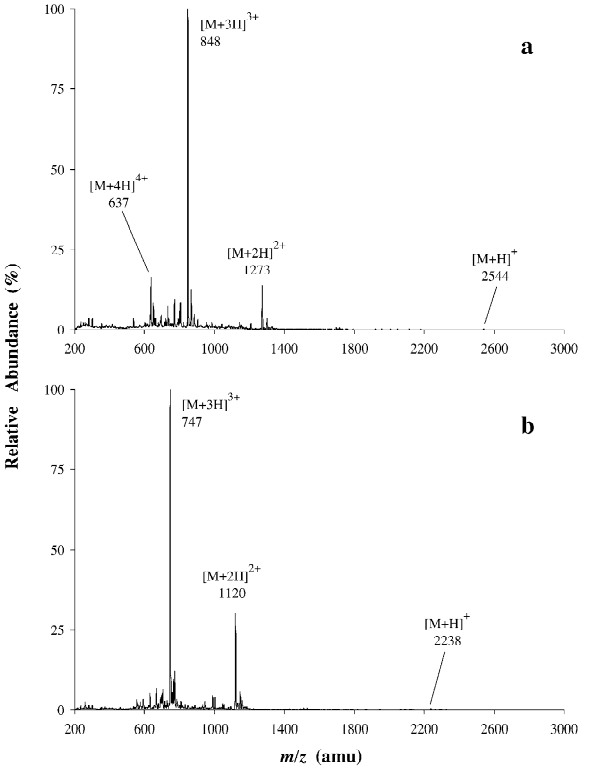

Fig. 4. Mass spectra of (a) peptide E1 and (b) peptide E2 obtained during on-line LC–ESI-MS analysis of digested rOaGST (see Fig. 3). Spectra correspond to the rOaGST 1–20 residues (a) with and (b) without bounded GSH (see Table 1).

on the Cys10 residue has been determined from the crystal structure of *Proteus mirabilis* GST [13].

The difference in the retention times of the two chromatograms (Fig. 3) was due to the elution effect of 2-ME (2%) in the microbore column, but this effect disappeared within 30 min.

3.3. LC–ESI-MS analyses of bacterial extracts

LC–ESI-MS analyses of crude bacterial extracts from *E. coli* overexpressing rOaGST were performed in order to verify the presence of the disulphide bond also in the non-purified enzyme. Although proteins and peptides chromatograms usually show a better resolution when trifluoroacetic acid (TFA) is used as mobile phase additive (due to its ion-pairing properties), TFA may inhibit the ESI-MS of the analyte because of competitive ionisation [31]. Formic acid (0.1–0.5%) can also be used as mobile phase additive in ESI-MS, achieving a sensitivity at least 30 times greater [32]. On the other hand, formic acid does not always give a satisfactory chromatographic resolution, as for instance, when horse heart myoglobin, here used as protein mass calibrator, was analysed by LC–ESI-MS (data not shown). In our case, rOaGST was chromatographed with a good resolution also with formic acid (Fig. 5a) and this allowed us to enhance the sensitivity of the analysis. No significant differences were observed in the retention times and in the chromatographic profiles of non-reduced and reduced intact purified rOaGST. As shown in Fig. 5a, the LC–ESI-MS analysis of the crude bacterial extract containing about 30% of rOaGST (as determined by SDS–PAGE [26]) has revealed a major peak having the same retention time of that of the purified rOaGST. The corresponding spectrum and its mass reconstruction (Fig. 5b) have shown a M_r corresponding to the non-glutathiolated form of rOaGST. The results shown in Fig. 5 were also obtained after incubation of *E. coli* crude extract with 10 and 50 m*M* GSH for 16 h at 4 °C (tempera-

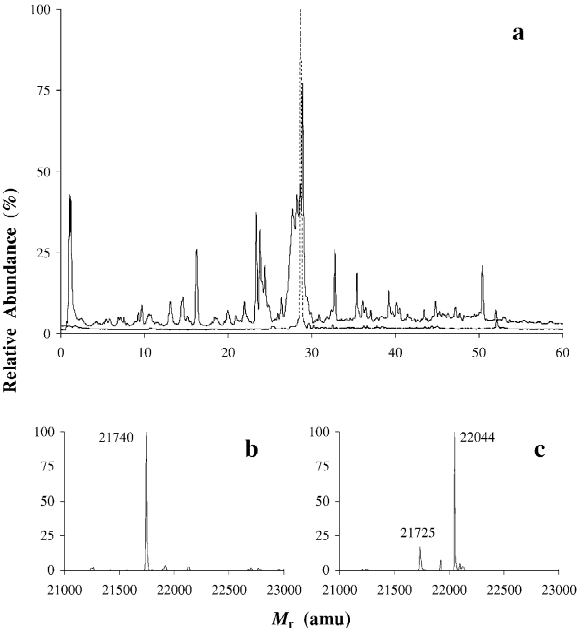

Fig. 5. (a) Total ion chromatograms of rOaGST before (unbroken line) and after (dotted line) purification with GSH-affinity chromatography; (b,c) reconstructed M_r of rOaGST in bacterial extract and after GSH-affinity chromatography, respectively.

ture of rOaGST purification procedure) and at room temperature (20–22 °C). On the other hand, the purified rOaGST has shown a predominant M_r corresponding to the *S*-glutathiolated form. These results may suggest that the *S*-glutathiolation of rOaGST may be due, or at least favoured, by GSH/GSH-affinity column/rOaGST interactions during the rOaGST purification procedure. Although the nature of these interactions remains unclear, a hypothetical mechanism of disulphide exchange should be excluded since rOaGST cysteines are in a free form. This was demonstrated by the double alkylation of the two cysteine residues when the protein was incubated with 25 m*M* iodoacetamide (in 100 m*M* NaOH) for 20 min at room temperature in the dark (data not shown).

4. Conclusions

In this study, we have described an ESI-MS method that enabled us to determine the presence of a *S*-glutathiolation on the Cys10 residue of rOaGST. This modification has been observed only when the enzyme was purified through a GSH-affinity column chromatography. It can be speculated that the disulphide bond formed between GSH and rOaGST is promoted during the purification step in which GSH is present both in the free form as eluting agent and bonded to the sepharose gel. In agreement with a previous study of Chen and co-workers [18], our results suggest that alternative GST purification systems should be used in order to discriminate naturally occurring modifications from artefacts, with the aim to better understand the role of *S*-glutathiolation in the regulation mechanisms of GSTs.

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