Cloning and expression of a single-chain catalytic antibody that acts as a glutathione peroxidase mimic with high catalytic efficiency

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Glutathione peroxidase (GPX) has a powerful role in scavenging reactive oxygen species. In previous papers we have developed a new strategy for generating abzymes: the monoclonal antibody with a substrate-binding site is first prepared, then a catalytic group is incorporated into the monoclonal antibody's binding site by using chemical mutation [Luo, Zhu, Ding, Gao, Sun, Liu, Yang and Shen (1994) *Biochem. Biophys. Res. Commun.* **198**, 1240–1247; Ding, Liu, Zhu, Luo, Zhao and Ni (1998) *Biochem. J.* **332**, 251–255]. Since then we have established a series of catalytic antibodies capable of catalysing the decomposition of hydroperoxides by GSH. The monoclonal antibody 2F3 was raised against GSH-S-2,4-dinitrophenyl *t*-butyl ester and exhibited high catalytic efficiency, exceeding that of rabbit liver GPX, after chemical mutation. To produce pharmaceutical proteins and to study the reason why it exhibits high catalytic efficiency, we sequenced, cloned and expressed the variable regions of 2F3 antibody as a single-chain Fv fragment (2F3-

scFv) in different bacterial strains. The amounts of 2F3-scFv proteins expressed from JM109 (DE3), BL21 (DE3), and BL21 (codon plus) were 5–10 %, 15–20 % and 25–30 % of total bacterial proteins respectively. The 2F3-scFv was expressed as inclusion bodies, purified in the presence of 8 M urea by Co²⁺-immobilized metal-affinity chromatography (IMAC) and renatured to the active form *in vitro* by gel filtration. The binding constants of the active 2F3-scFv for GSH and GSSG were $2.46 \times 10^5 \text{ M}^{-1}$ and $1.03 \times 10^5 \text{ M}^{-1}$ respectively, which were less by one order of magnitude than that of the intact 2F3 antibody. The active 2F3-scFv was converted into selenium-containing 2F3-scFv (Se-2F3-scFv) by chemical modification of the reactive serine; the GPX activity of the Se-2F3-scFv was 3394 units/ μmol , which approaches the activity of rabbit liver GPX.

Key words: artificial enzyme, chemical mutation, enzyme mimics, protein engineering, selenium.

INTRODUCTION

Reactive oxygen species, including the superoxide anion (O_2^-), H_2O_2 , organic peroxides and the hydroxyl radical, are normal products of aerobic metabolism. However, they are known to destroy key biological molecules and cause damage to cell membranes [1]. Examples of such oxidative-stress-related diseases include reperfusion injury, brain ischaemia, tumour, cataract and various types of inflammation and physiological aging. Living organisms have evolved a family of antioxidant enzymes to cope with oxidative stress, including superoxide dismutase, which catalyses the dismutation of O_2^- to H_2O_2 , the selenium-containing enzyme glutathione peroxidase (GPX), which catalytically destroys hydroperoxides, and catalase, which catalyses the breakdown of H_2O_2 . The GPXs are substantially more efficient on a molar basis than the other enzymes [2].

At least four forms of GPX have been reported so far; they are different in many properties, including their localization, subunit structure, primary structure and enzymic nature. The classical cellular GPX (GSHPx-1), found in the cytosol of various tissues and blood cells, reduces H_2O_2 and organic hydroperoxides but cannot reduce phospholipid hydroperoxides [3]. A second GPX, phospholipid hydroperoxide GPX, is believed to be mostly cytosolic and partly membrane-bound and reduces phospholipid hydroperoxide as well as H_2O_2 [4–6]. A third GPX in plasma, extracellular GPX, is reported to reduce both H_2O_2 and phospholipid hydroperoxides [7,8]. A fourth GPX, gastrointestinal GPX,

is highly expressed in the mucosal epithelium of the gastrointestinal tract [9,10]. Of these forms, GSHPx-1 has been well studied [3]. The mechanism by which GSHPx-1 catalyses the breakdown of hydroperoxides has been extensively studied (Scheme 1). The selenol of a reduced selenocysteine molecule (ESeH) is oxidized by the hydroperoxides to generate a selenenic acid (ESeOH). The tripeptide GSH then reacts with the selenenic acid, affording the corresponding water and selenenyl

Scheme 1 Mechanism by which GSHPx-1 catalyses the breakdown of hydroperoxides

Abbreviations used: 2F3-scFv, single-chain Fv fragment of monoclonal antibody 2F3; GPX, glutathione peroxidase; GSHPx-1, classical cellular GPX; IMAC, immobilized metal-affinity chromatography; IPTG, isopropyl β -D-thiogalactoside; N₂ph, 2,4-dinitrophenyl; scFv, single-chain Fv fragment; Se-2F3-scFv, selenium-containing 2F3-scFv.

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Figure 1 Structures of the haptens used to generate antibodies

From top left, clockwise: GSH-S-N₂phMe, 3G5; GSH-S-N₂phBu, 2F3; GSSGMe, 5C9; GSH-S-N₂ph, 4A4.

Table 1 GPX activities of Se-2F3-scFv compared with other catalytic antibodies and native enzyme

Reactions were performed in 50 mM potassium phosphate buffer, pH 7.0, at 37 °C, containing 1 mM GSH and 0.5 mM H₂O₂. Results are presented as means ± S.D.

Species	Specific GPX activity (units/μmol)	GPX activity (units/mg)
Se-4A4	1097 ± 22	7.3 ± 0.2
Se-3G5	12 900 ± 390	86 ± 3
Se-5C9	21 900 ± 652	146 ± 4
Se-2F3	24 300 ± 729	162 ± 5
Se-2F3-scFv	3394 ± 68	114 ± 3
Native GPX (rabbit liver)	5780	85.7

sulphide (ESeSG). A second molecule of GSH attacks the sulphur of the latter species, producing GSH disulphide and regenerating the selenol (ESeH) to complete the catalytic cycle [3].

Recently, many GPX mimics have been prepared and have been used to study antioxidant activity. In particular, the cyclic selenenamide Ebselen has been shown to be a good catalyst for the reduction of peroxides and hydroperoxides with thiol [11]. Although this interesting molecule has been researched extensively from pulse-radiolytic studies on radical reactivity through its biological properties in organs to phase III clinical trials in Japan as an antioxidant [12–15], it has some drawbacks such as its low GPX activity and its insolubility in water [12]. In addition to selenocyclodextrins [16–18], other mimics have been well reviewed in [19].

Complementarity between enzyme and transition state is the essence of biological catalysis, which has been used to generate catalytic antibody [20–22]. The reaction mechanism of native GPX is very complicated and proceeds through several intermediates, which have not been fully explained and therefore the transition state is not known; thus a reasonable transition-state analogue has not been available. Using the substrate analogues GSH-S-N₂ph (4A4) [23,24], GSH-S-N₂phMe (3G5), GSSGMe (5C9) and GSH-S-N₂phBu (2F3) [25] as haptens (in which N₂ph represents 2,4-dinitrophenyl) (Figure 1), we obtained monoclonal antibodies capable of binding the substrate GSH. These monoclonal antibodies could be converted into catalytic antibodies when the selenocysteine residues were incorporated into the binding sites of the antibodies. In this way we generated several

catalytic antibodies that efficiently catalysed the breakdown of hydroperoxides by GSH. Among them, several antibodies have been found to display remarkably high catalytic efficiencies, which exceed those of some of the native enzymes (Table 1). In the present study we constructed an expression vector of 2F3-scFv, the single-chain Fv (scFv) fragment of monoclonal antibody 2F3, for the large-scale production of recombinant protein as inclusion bodies in bacteria. The refolded scFv protein was converted into selenium-containing 2F3-scFv (Se-2F3-scFv) protein and the GPX activity of the Se-2F3-scFv-catalysed reduction of H₂O₂ by GSH approached the activity of GPX from rabbit liver.

EXPERIMENTAL

Materials

Plasmids pTMF, pTHA90 and pGEM-T and *Escherichia coli* JM109 (DE3), BL21 (DE3) and BL21 (codon plus) were provided by Dr Hualiang Huang (Institute of Genetics, Chinese Academy of Sciences, Beijing, China). The *E. coli* Top10 was purchased from Invitrogen. Oligonucleotides were synthesized with an Applied Biosystems 391 DNA synthesizer and were purified on oligonucleotide purification cartridges (Applied Biosystems). Chelating Sepharose Fast Flow, Sephadex G-75 and Sephadex G-25 were purchased from Pharmacia Biotech. Ultrogel AcA44 was obtained from LKB. All other chemicals were of analytical grade and were readily available from commercial sources.

Electrophoresis and Western blotting

SDS/PAGE was run with a Pharmacia PhastSystem with 12.5% (w/v) gels. Gels were stained with Coomassie Brilliant Blue G250. The proteins produced in the *E. coli* cultures were subjected to SDS/PAGE, blotted to nitrocellulose membrane and incubated with blocking buffer at room temperature for 1 h. The membrane was incubated with anti-His₆ antibody and then peroxidase-conjugated goat anti-mouse IgG. Finally, peroxidase signals were measured with a 3,3',5,5'-tetramethylbenzidine system.

Cloning of antibody variable genes

Hybridomas were prepared from mice immunized with a hapten, GSH-S-N₂phBu (2F3), conjugated with BSA. Total RNA was purified from hybridomas with a Total RNA Extraction System

(Promega); mRNA was isolated from total RNA with a PolyAT Tract System (Promega) and cDNA was generated by reverse-transcriptase-mediated PCR by using Time Saver cDNA Synthesis (Promega). PCR amplification of V_H and V_L was performed with the light-chain primer mix (Pharmacia Biotech) for V_L and the heavy-chain primer mix (Pharmacia Biotech) for V_H , for 30 cycles of 94 °C for 1 min, 55 °C for 2 min and 72 °C for 1 min. This was followed by a single incubation at 72 °C for 10 min. PCR products were purified by extraction with phenol/chloroform (25:24, v/v) and precipitation with ethanol, ligated to the plasmid vector pGEM-T and transformed into *E. coli* Top 10.

Construction of a 2F3-scFv expression vector

The plasmids containing the cloned heavy and light variable regions were used as templates for PCR with primers V_L 5' (5'-CCTCTAGAGCTGTTGTGATGACCCAACTCC-3'; *Xba*I) and V_L 3' (5'-CCGAATTCTTTCATTTCAGCTTGGTCCCG-3'; *Eco*RI) for V_L , and V_H 5' (5'-GGCTCGAGCAGGTGCAGCTGCAGCAGTCAGG-3'; *Xho*I) and V_H 3' (5'-GGACTAGT TGAGGAGACGGTGACCGTGGTCC-3'; *Spe*I) for V_H . PCR was performed with 30 cycles (denaturation at 94 °C for 1 min; annealing at 60 °C for 1 min and extension at 72 °C for 1 min). The PCR products were isolated by agarose-gel electrophoresis and purified with a Watson gel extraction kit (Promega), then cleaved with relevant restriction enzymes (Promega) and cloned separately into pTHA90 vector. Finally, V_H genes and V_L genes were incorporated into the same vector and connected with a linker (Gly₄Ser)₃. The resulting plasmid was designated pTHA90scFv. Colonies selected from the pTHA90scFv construct were used to confirm insert integrity by restriction-enzyme digestion and PCR analysis. DNA sequencing was then performed in the DNA Sequencing Unit of the Genecore Corporation of Shanghai by Applied Biosystems DNA sequencers with the T7 Sequencing[®] kit (Pharmacia).

The pTHA90scFv construct was digested with the relevant restriction enzymes (*Eco*RI and *Xba*I) and ligated into pTMF expression vector that had been digested with the same restriction enzyme. The resulting plasmid encoding scFv was designated pTMFscFv, which was used to transform *E. coli* Top 10. After transformation, colonies were selected in which the pTMFscFv construct was in the correct orientation, as judged by restriction mapping and PCR analysis.

Expression of 2F3-scFv gene in *E. coli*

E. coli JM109 (DE3), BL21 (DE3) and BL21 (codon plus) cells were transformed with pTMFscFv containing the V_H -linker- V_L construct and each cultivar was grown at 37 °C to mid-exponential phase (D_{590} 0.5–1.0) in Luria-Bertani medium containing kanamycin (50 µg/ml). The production of 2F3-scFv proteins was then induced by the addition of 1 mM isopropyl β-D-thiogalactoside (IPTG). After incubation for 8 h, the pellets from 2 ml cultures from a single colony were lysed in SDS loading buffer and subjected to SDS/PAGE. One colony that produced large amounts of protein as judged by Coomassie staining was chosen for further experiments. For the large-scale production of 2F3-scFv proteins this colony was grown and induced as described above in 500 ml. The cell pellet was washed with TE buffer [20 mM Tris/2.5 mM EDTA (pH 8.0)] and resuspended at a density of 1 g of wet bacteria per 10 ml of TE containing hen's-egg lysozyme (1 mg/ml). After incubation overnight at 4 °C, cell lysis was completed by sonication in the presence of 0.5% (v/v) Triton X-100. Inclusion bodies were pelleted at 22000 g for 30 min and washed twice with TE buffer containing 0.5% Triton

X-100, followed by three more washes with TE buffer without Triton X-100.

Purification and refolding of 2F3-scFv from inclusion bodies

Inclusion bodies were purified by Co²⁺-immobilized metal-affinity chromatography (IMAC) before being refolded. In brief, inclusion bodies were resuspended in buffer TU [20 mM Tris/HCl/8 M urea/0.01 M NaOH (pH 8.0)] at 5 ml/g wet weight, then stirred for 1 h at room temperature. The soluble fraction was collected after centrifugation at 10000 g for 15 min, loaded on a Co²⁺-IMAC column (2 ml of gel/1 of culture) previously equilibrated with buffer I [20 mM Tris/HCl/8 M urea (pH 8.0)] and washed with buffer II [20 mM Tris/HCl/10 mM imidazole/0.5 M NaCl/8 M urea (pH 8.0)]; bound 2F3-scFv was eluted with buffer III [20 mM Tris/HCl/100 mM imidazole/0.5 M NaCl/8 M urea (pH 8.0)].

The refolding of inactive 2F3-scFv was performed by gel-filtration chromatography as follows. The fraction eluted from buffer III was loaded on an Ultrogel AcA44 column (1 cm × 90 cm) previously equilibrated with buffer IV [20 mM Tris/HCl/1 mM urea (pH 8.0)] and refolded with buffer V [20 mM Tris/HCl/1 M urea/0.5 mM GSH/0.1 mM GSSG (pH 8.0)]. While the column was running, the flow rate was 0.2 ml/min. The refolded fractions were pooled, loaded on a Sephadex G-75 column previously equilibrated with buffer VI (20 mM Tris/HCl, pH 8.0) and eluted with buffer VI. The first peak was collected and analysed by SDS/PAGE. The yield of the protein at this stage varied between 6 and 10 mg from 1 litre of bacterial culture, as determined by A_{280} .

Measurement of the binding constant of active 2F3-scFv for GSH and GSSG

The binding constant of the active 2F3-scFv for GSH was assessed by the quenching of 2F3-scFv tryptophan fluorescence with GSH as described [26]. Refolded samples were adjusted to an A_{280} of less than 0.05 in a final volume of 2 ml with buffer (0.1 M Tris/HCl, pH 7.5). Fluorescence was measured on a Hitachi 850 spectrofluorometer with excitation at 295 nm and emission at 333 nm. Samples were quenched with GSH, the concentration of which was gradually increased in a titration range. The binding constant of the active 2F3-scFv for GSSG was measured as described above.

Preparation of Se-2F3-scFv

The active 2F3-scFv proteins (0.1 mg) were dissolved in 1 ml of the solution containing 50 mM potassium phosphate, pH 7.0, and allowed to react with PMSF (4 µl of 0.2 mg/ml in acetonitrile) at 25 °C for 1 h. Nitrogen gas was bubbled into the mixture for 20 min, mixed with 10 µl of 1 M NaHSe solution prepared by the procedure of Klayman and Griffin [27] and then incubated at 40 °C for 36 h under a nitrogen atmosphere. The proteins were separated from the reaction mixture on a Sephadex G-25 column eluted with buffer (20 mM Tris/HCl, pH 8.0). The active fractions were pooled and finally freeze-dried.

Determination of the GPX activity of Se-2F3-scFv

The GPX activity of Se-2F3-scFv was assayed as described in [3]. The reaction was performed at 37 °C in 500 µl of a solution containing 50 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM Na₂S₂O₃, 1 mM GSH, 1 unit of GSH reductase and 10–50 nM Se-2F3-scFv. The mixture was preincubated for 7 min;

0.25 mM NADPH solution was then added and incubated for 3 min at 37 °C. Thereafter the reaction was initiated by the addition of 0.5 mM H₂O₂. The activity was determined by the decrease in NADPH absorption at 340 nm. Appropriate controls were run without Se-2F3-scFv and subtracted. The activity unit of Se-2F3-scFv is defined as the amount of Se-2F3-scFv that uses 1 µmol of NADPH/min. Specific activity is expressed in units/µmol of Se-2F3-scFv.

RESULTS

Construction of expression vector

The genetic construct used to generate a 2F3-scFv from mice hybridomas immunized with a hapten, GSH-S-N₂phBu (2F3), conjugated to BSA is shown in Scheme 2. The construct encodes the V_H fragment of antibody 2F3 followed by a spacer (Gly₄Ser)₃ that links it to the C-terminus of the V_L fragment. The linker consists of 15 residues and has commonly been used in constructing scFv protein from an antibody. In addition, the construct encodes a hexahistidine tag at the C-terminus of the V_L fragment. The sequences of the V_H and V_L inserts are shown in Figure 2. Repeated amplification, cloning and sequencing of V_H

Scheme 2 Protocol for the construction of the expression vector pTMFscFv encoding 2F3-scFv nucleotide sequences

V_H:

1	Q	V	Q	L	Q	Q	S	G	P	E	L	V	K	T	G	A	16
I	C	A	G	T	G	C	A	G	C	T	A	G	C	T	A	G	48
17	S	V	K	I	S	C	K	A	S	G	Y	S	F	T	<u>G</u>	<u>Y</u>	32
49	T	C	A	G	T	A	G	A	T	C	T	G	C	A	G	G	96
33	<u>Y</u>	<u>M</u>	<u>H</u>	<u>W</u>	<u>V</u>	<u>M</u>	<u>Q</u>	<u>S</u>	<u>H</u>	<u>G</u>	<u>K</u>	<u>S</u>	<u>L</u>	<u>E</u>	<u>W</u>	<u>I</u>	48
97	T	A	C	A	G	T	A	G	C	A	T	G	A	G	C	T	144
CDR1																	
49	G	<u>Y</u>	<u>I</u>	<u>S</u>	<u>C</u>	<u>Y</u>	<u>N</u>	<u>G</u>	<u>A</u>	<u>T</u>	<u>S</u>	<u>Y</u>	<u>N</u>	<u>Q</u>	<u>M</u>	<u>F</u>	64
145	G	A	T	A	T	A	G	T	T	A	C	A	T	G	T	T	192
CDR2																	
65	<u>K</u>	<u>D</u>	<u>K</u>	<u>A</u>	<u>T</u>	<u>F</u>	<u>T</u>	<u>V</u>	<u>N</u>	<u>S</u>	<u>S</u>	<u>S</u>	<u>S</u>	<u>T</u>	<u>T</u>	<u>F</u>	80
193	A	A	G	A	G	C	A	T	T	A	C	T	A	C	A	T	240
81	M	Q	F	N	S	L	T	S	E	D	S	A	V	Y	Y	C	96
241	A	T	G	C	A	T	A	C	C	T	G	A	A	G	C	T	288
97	A	R	<u>C</u>	<u>G</u>	<u>Y</u>	<u>Y</u>	<u>V</u>	<u>G</u>	<u>F</u>	<u>A</u>	<u>Y</u>	<u>W</u>	<u>G</u>	<u>Q</u>	<u>G</u>	<u>T</u>	112
289	G	C	A	G	T	G	T	A	C	T	T	G	C	A	G	G	336
CDR3																	
113	T	V	T	V	S	S											118
337	A	C	G	T	C	A	T	T	C	A							354

V_L:

1	A	V	V	M	T	Q	T	P	L	S	L	P	V	S	L	G	16
1	G	C	T	G	T	A	T	C	C	A	C	T	C	C	T	G	48
17	D	Q	A	S	I	S	C	<u>R</u>	<u>S</u>	<u>S</u>	<u>Q</u>	<u>S</u>	<u>L</u>	<u>V</u>	<u>H</u>	<u>S</u>	32
49	G	A	T	C	A	G	C	T	A	T	C	T	G	A	C	A	96
CDR1																	
33	<u>N</u>	<u>G</u>	<u>N</u>	<u>T</u>	<u>Y</u>	<u>L</u>	<u>H</u>	<u>W</u>	<u>Y</u>	<u>L</u>	<u>Q</u>	<u>K</u>	<u>P</u>	<u>G</u>	<u>Q</u>	<u>S</u>	48
97	A	A	T	A	C	A	T	T	A	C	T	G	A	A	G	C	144
49	P	K	L	L	I	Y	<u>K</u>	<u>V</u>	<u>S</u>	<u>N</u>	<u>R</u>	<u>F</u>	<u>S</u>	<u>G</u>	<u>V</u>	<u>P</u>	64
145	C	A	A	G	C	T	G	A	T	T	C	A	A	G	T	T	192
CDR2																	
65	D	R	F	S	G	S	G	S	T	D	F	T	L	K	I		80
193	G	A	G	T	T	A	G	T	G	A	C	A	G	A	T	C	240
81	S	R	V	E	A	E	D	L	G	V	Y	F	C	<u>S</u>	<u>Q</u>	<u>S</u>	96
241	A	C	A	G	T	G	C	T	G	A	T	T	T	T	G	C	288
97	<u>T</u>	<u>H</u>	<u>V</u>	<u>P</u>	<u>Y</u>	<u>T</u>	<u>F</u>	<u>G</u>	<u>G</u>	<u>G</u>	<u>T</u>	<u>K</u>	<u>L</u>	<u>E</u>	<u>I</u>	<u>K</u>	112
289	A	C	A	T	G	T	C	C	A	G	T	T	C	A	A		336
CDR3																	
113	R																113
337	C	G															339

Figure 2 DNA and amino acid sequences of V_H and V_L

Figure 3 Agarose-gel electrophoresis of V_H and V_L PCR products

Lane 1, PCR product of 2F3V_H; lane 2, PCR product of 2F3V_L; lane 3, molecular mass markers.

and V_L indicated that our sequence is correct and that there was no PCR error (Figure 3).

Expression and partial purification of recombinant 2F3-scFv

Bacteria containing the encoding 2F3-scFv sequences in the pTMF vector produce a significant fraction of scFv (29.9 kDa) after induction with IPTG (Figure 4A). The amounts of 2F3-scFv proteins expressed in *E. coli* JM109 (DE3), BL21 (DE3) and BL21 (codon plus) were 5–10 %, 15–20 % and 25–30 % of total

Figure 4 SDS/PAGE and Western-blot analysis of 2F3-scFv

(A) SDS/PAGE analysis of 2F3-scFv at different stages of each step. Lane 1, molecular mass markers (values given at the left, in kDa); lane 2, total bacterial lysate; lane 3, inclusion bodies; lane 4, 2F3-scFv purified through IMAC; lane 5, renatured 2F3-scFv; lane 6, renatured 2F3-scFv purified through Sephadex G-75. (B) Western blotting analysis of 2F3-scFv expressed protein. Lane 1, molecular size marker (value indicated at the left, in kDa); lane 2, uninduced with IPTG; lane 3, inclusion bodies; lane 4, total bacterial lysate.

bacterial proteins respectively. Western blots of SDS/PAGE gels were probed with anti-His₆ and then with peroxidase-conjugated goat anti-mouse IgG. As shown in Figure 4(B), the anti-His₆ cross-reacted with the recombinant 2F3-scFv in both inclusion bodies and total bacteria lysate, indicating that 2F3-scFv proteins were expressed in the bacteria. The 2F3-scFv proteins were partly purified by washing the bacterial pellet with a detergent solution, thus taking advantage of the insolubility of the protein in inclusion bodies. This procedure produced recombinant proteins that were at least 50% pure (Figure 4A).

There are many previous examples of purification, by IMAC, of proteins with His tags expressed in *E. coli* [28]. These chromatographic steps all operated with buffers that kept the proteins in the denatured reduced state. The loaded samples were eluted with two different buffers; samples containing the second of the two peaks were pooled.

Refolding of the recombinant 2F3-scFv *in vitro*

Inclusion bodies were solubilized in 8 M urea; disulphide bonds were fully reduced. Starting with totally reduced and denatured material, mixed disulphide bonds were formed between the thiol groups of the protein and GSH; the modified 2F3-scFv was allowed to gain its native conformation by gel filtration on an

Ultrogel Ac4A4 column loaded with renaturation buffer containing 0.5 mM GSH and 0.1 mM GSSG, under conditions that allowed the reshuffling of S–S bonds. After refolding *in vitro*, most of the recombinant protein migrated on SDS/PAGE more rapidly than the reduced protein (Figure 4A), showing that intramolecular disulphide bonds had formed [29]. The refolded native proteins were further purified with a Sephadex G-75 column.

Determination of the binding constant of 2F3-scFv and the GPX activity of Se-2F3-scFv

The fluorescence quench assay and the calculation of affinity were performed for 2F3-scFv. Values were calculated from Scatchard plots [22] as $(2.46 \pm 0.21) \times 10^5 \text{ M}^{-1}$ and $(1.03 \pm 0.14) \times 10^5 \text{ M}^{-1}$ for GSH and GSSG respectively (means $\pm$ S.D.). The binding constants of intact antibody 2F3 were $2.91 \times 10^6 \text{ M}^{-1}$ and $9.24 \times 10^5 \text{ M}^{-1}$ for GSH and GSSG respectively.

Active serine residues in the antibody variable regions can be converted into selenocysteine residues by chemical mutation. Therefore the essential catalytic selenocysteine residues can also be incorporated into the GSH-binding site of active 2F3-scFv. The method for preparing Se-2F3-scFv was the same as that reported for preparing selenium-containing antibody enzyme [21,22]. The GPX activity of the Se-2F3-scFv-catalysed reduction of H₂O₂ by GSH was measured with a coupled test procedure assay [3] and found to be 3394 units/ μmol , which approaches the GPX activity of native enzyme from rabbit liver.

DISCUSSION

Although some catalytic antibodies have been reported with efficiencies similar to that of the corresponding natural enzyme, in general it has been difficult to obtain a highly active catalyst [30]. We established a series of catalytic antibodies capable of catalysing the breakdown of H₂O₂ by GSH with different haptens. A group of these catalytic antibodies exhibit high reactivity and substrate specificity for GSH, yet each individual antibody shows different kinetic parameters [25]. Most importantly, some of these catalytic antibodies have catalytic efficiencies that remarkably exceed that of the native enzyme (Table 1).

In the present study we produced an scFv recombinant protein from 2F3 hybridoma cells. We choose this particular 2F3 antibody because its GPX activity was the highest. The restriction enzyme sites of encoding V_L and V_H sequences in pGEM-T vector were not suitable for the pTHA90 vector. PCR amplification was therefore performed to introduce restriction enzyme sites into the V_L and V_H sequences by using appropriate primers. The sequences that encoded V_L and V_H were subcloned into pTHA90 and linked with a linker (Gly₄Ser)₃ belonging to pTHA90. However, the quantity of 2F3-scFv proteins produced by the pTHA90 vector was small. The sequences that encoded (NH₂)₂-V_H-linker-V_L-COOH were subcloned into pTMF expression vector, which uses the bacteriophage T7 RNA polymerase and the T7 promoter to drive protein synthesis. The T7 polymerase elongates polypeptide chains approx. 5-fold faster than the endogenous bacterial polymerase [31]. The rapid production of such material is the major advantage of using bacterial inclusion bodies to produce scFv. When the pTMF expression vector was transformed into the different bacterial strains JM109 (DE3), BL21 (DE3) and BL21 (codon plus), the amounts of scFv proteins produced were different. The differences perhaps relate to the genetic background and cytosolic environment of the strains.

The main problem that we encountered during the production of active 2F3-scFv was in the refolding process. The abundance

of protein expression systems renders the efficient production of most proteins possible but high-level expression of the recombinant proteins often results in protein aggregation and the accumulation of inclusion bodies [29]. Although several methods, including dilution, dialysis, diafiltration, gel filtration and immobilization on a solid support, can be employed to remove or decrease the levels of excess denaturing and reducing agents, allowing proteins to renature, each method is effective for an individual inclusion body [32]. The inactive 2F3-scFv protein was refolded with the use of gel filtration but the refolding yield was very low. The 2F3-scFv was produced in bacteria as inclusion bodies and approx. 6–10 mg of protein from 1 litre of bacterial culture was obtained after refolding.

The binding activity of the active 2F3-scFv was lower than that of the intact 2F3 antibody. Bacterially produced 2F3-scFv fragment usually has a lower affinity than the native, intact antibody from which it is derived, presumably owing to incorrect folding [33,34]. The binding constants of 2F3 antibody for GSH and GSSG were about 10-fold that of the active 2F3-scFv by fluorescence quench assay, indicating that 2F3-scFv retains the conformation of the variable regions of 2F3. The GPX activity of the Se-2F3-scFv-catalysed reduction of H_2O_2 by GSH was 3394 units/ μ mol, which approaches that of the native enzyme from rabbit liver but was lower than that of the selenium-containing abzyme 2F3, which was 24300 units/ μ mol. These results mean that pTMFscFv expression vector was correctly constructed and expressed in *E. coli*.

In conclusion, we have successfully constructed pTMFscFv expression vector. The 2F3-scFv protein produced by *E. coli* containing 2F3-scFv expression vector retains the binding and catalytic capabilities of the parent catalytic antibody. The expression of a catalytic antibody in single-chain form offers several advantages. The ability of the protein to be expressed in large amounts in *E. coli* may be advantageous for further large-scale applications of catalytic antibodies and structural and biochemical studies. The smaller size and lower antigenicity of 2F3-scFv protein makes it have a great potential in the treatment of diseases mediated by oxidative stress.

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REFERENCES

- Vaughan, M. (1997) Oxidative modification of macromolecules minireview series. *J. Biol. Chem.* **272**, 18513
- Michiels, C., Raes, M., Toussaaint, O. and Remacle, J. (1994) Importance of Se-glutathione peroxidase, catalase, and Cu/Zn-SOD for cell survival against oxidative stress. *Free Radical Biol. Med.* **17**, 235–248
- Flohé, L. (1989) The selenoprotein glutathione peroxidase. In *Glutathione: Chemical, Biochemical, Medical Aspects* (Dolphin, D., Poulsen, R. and Avramovic, O., eds.), pp. 643–731. John Wiley, Sons, New York
- Roveri, A., Maiorino, M., Nisini, C. and Ursini, F. (1994) Purification and characterization of phospholipid hydroperoxide glutathione peroxidase from rat testis mitochondrial membranes. *Biochim. Biophys. Acta* **1208**, 211–221
- Pushpa-Rekha, T. R., Burdsau, A. L., Oleksa, L. M., Chisolm, G. M. and Driscoll, D. M. (1995) Rat phospholipid-hydroperoxide glutathione peroxidase. cDNA cloning and identification of multiple transcription and translation start sites. *J. Biol. Chem.* **270**, 26993–26999
- Thomas, J. P., Maiorino, M., Ursini, F. and Girutti, A. W. (1990) Protective action of phospholipid hydroperoxide glutathione peroxidase against membrane-damaging lipid peroxidation. In situ reduction of phospholipid and cholesterol hydroperoxides. *J. Biol. Chem.* **265**, 454–461
- Takahashi, K., Avissar, N., Whitin, J. and Cohen, H. (1987) Purification and characterization of human plasma glutathione peroxidase: a selenoglycoprotein distinct from the known cellular enzyme. *Arch. Biochem. Biophys.* **256**, 677–686
- Yamamoto, Y. and Takahashi, K. (1993) Glutathione peroxidase isolated from plasma reduces phospholipid hydroperoxides. *Arch. Biochem. Biophys.* **305**, 541–545
- Akasaka, M., Mizoguchi, J. and Takahashi, K. (1990) A human cDNA sequence of a novel glutathione peroxidase-related protein. *Nucleic Acids Res.* **15**, 4619
- Esworthy, R. S., Swiderek, K. M., Ho, Y. S. and Chu, F. F. (1998) Selenium-dependent glutathione peroxidase-GI is a major glutathione peroxidase activity in the mucosal epithelium of rodent intestine. *Biochim. Biophys. Acta* **1381**, 213–226
- Müller, A., Cadenas, E., Graf, P. and Sies, H. (1984) A novel biologically active selenoorganic compound. I. Glutathione peroxidase-like activity in vitro and antioxidant capacity of PZ51 (Ebselen). *Biochem. Pharmacol.* **33**, 3235–3239
- Sies, H. (1993) Ebselen, a selenoorganic compound as glutathione peroxidase mimic. *Free Radical Biol. Med.* **14**, 313–323
- Sies, H. (1994) Ebselen, a glutathione peroxidase mimic. *Methods Enzymol.* **234**, 476–482
- Sies, H. (1997) Ebselen as a glutathione peroxidase mimic and as scavenger of peroxynitrite. *Adv. Pharmacol.* **38**, 229–246
- Sies, H. (2000) Interaction of peroxynitrite with selenoproteins and glutathione peroxidase mimics. *Free Radical Biol. Med.* **15**, 1451–1455
- Liu, J. Q., Gao, S. J., Luo, G. M., Yan, G. L. and Shen, J. C. (1998) Artificial imitation of glutathione peroxidase with 6-seleno-bridged β -cyclodextrin. *Biochem. Biophys. Res. Commun.* **247**, 397–400
- Liu, J. Q., Luo, G. M., Ren, X. J., Mu, Y., Bai, Y. and Shen, J. C. (2000) A-bis-cyclodextrin diselenide with glutathione peroxidase-like activity. *Biochim. Biophys. Acta* **1481**, 222–228
- Ren, X. J., Liu, J. Q., Luo, G. M., Zhang, Y., Luo, Y. M., Yan, G. L. and Shen, J. C. (2000) A-bis-cyclodextrin diselenide with glutathione peroxidase-like activity. *Bioconj. Chem.* **11**, 682–687
- Mughes, G. and Singh, H. B. (2000) Synthetic organoselenium compounds as antioxidants: glutathione peroxidase activity. *Chem. Soc. Rev.* **29**, 347–357
- Pauling, L. (1946) Molecular architecture and biological reactions. *Chem. Eng. News* **24**, 1375–1377
- Stewart, J. D. and Benkovic, S. J. (1995) Transition-state stabilization as a measure of the efficiency of antibody catalysis. *Nature (London)* **375**, 388–391
- Ulrich, H. D., Mundorff, E., Santarsiero, B. D., Driggers, E. M., Stevens, R. C. and Schultz, P. G. (1997) The interplay between binding energy and catalysis in the evolution of a catalytic antibody. *Nature (London)* **389**, 271–275
- Luo, G. M., Zhu, Z. Q., Ding, L., Gao, G., Sun, Q. A., Liu, Z., Yang, T. S. and Shen, J. C. (1994) Generation of selenium-containing abzyme by using chemical mutation. *Biochem. Biophys. Res. Commun.* **198**, 1240–1247
- Ding, L., Liu, Z., Zhu, Z. Q., Luo, G. M., Zhao, D. Q. and Ni, J. Z. (1998) Biochemical characterization of selenium-containing catalytic antibody as a cytosolic glutathione peroxidase mimic. *Biochem. J.* **332**, 251–255
- Su, D., Ren, X. J., You, D. L., Mu, Y., Yan, G. L., Luo, Y. M., Xue, Y., Shen, J. C., Liu, Z. and Luo, G. M. (2001) Generation of three selenium-containing catalytic antibodies with high catalytic efficiency using a novel hapten design method. *Arch. Biochem. Biophys.*, in the press
- Jolley, M. E. and Glaudemans, C. P. (1974) The determination of binding constants for binding between carbohydrate ligands and certain proteins. *Carbohydr. Res.* **33**, 377–381
- Klayman, D. L. and Griffin, T. S. (1973) Reaction of selenium with sodium borohydride in protic solvents. A facile method for the introduction of selenium into organic molecule. *J. Am. Chem. Soc.* **95**, 197–199
- Porath, J. (1992) Immobilized metal affinity chromatography. *Protein Expr. Purif.* **3**, 263–281
- Kurucz, I., Jost, C. R., George, A. J. T., Andrew, S. M. and Segal, D. M. (1993) A bacterially expressed single-chain Fv construct from the 2B4 T-cell receptor. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3830–3834
- Driggers, E. M. and Schultz, P. G. (1996) Catalytic antibodies. *Adv. Prot. Chem.* **49**, 261–287
- Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendorff, J. W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**, 60–89
- Clark, E. D. B. (1998) Refolding of recombinant proteins. *Curr. Opin. Biotechnol.* **9**, 157–163
- McGregor, D. P., Molloy, P. E., Cunningham, C. and Harris, W. J. (1994) Spontaneous assembly of bivalent single chain antibody fragments in *Escherichia coli*. *Mol. Immunol.* **31**, 219–226
- Navarro-Teulon, I., Peraldi-Roux, S., Bernardi, T., Marin, M., Piechaczyk, M., Shire, D., Pau, B. and Biard-Piechaczyk, M. (1995) Expression in *Escherichia coli* of soluble and M13 phage-displayed forms of a single-chain antibody fragment specific for digoxin: assessment in a novel drug immunoassay. *Immunotechnology* **1**, 41–52