Generation of a glutathione peroxidase-like mimic using bioimprinting and chemical mutation

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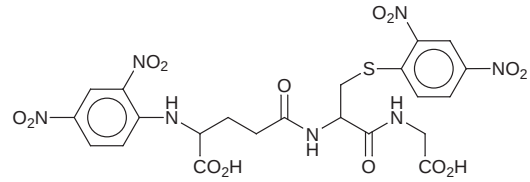
Molecular imprinting of albumin with *N*,*S*-bis(2,4-dinitrophenyl)glutathione (GSH-2DNP), a glutathione (GSH) derivative, leads to an imprinted protein with GSH binding sites; chemical mutation of the protein results in formation of an artificial enzyme with high glutathione peroxidase (GPX) activity.

The catalytic efficiency of an enzyme is dependent on its ability to recognise substrates, and the generation of recognition sites for substrates plays an important role in enzyme imitation.[1] In general, two strategies have been used to generate this recognition, the monoclonal antibody technique and molecular imprinting.[2,3] Glutathione peroxidase (GPX, EC 1.11.1.9) is an important antioxidative enzyme that catalyzes the reduction of a wide variety of hydroperoxides by glutathione (GSH).[4] The enzyme is composed of four identical subunits; the active site in each subunit contains a selenocysteine residue (catalytic group) and a GSH binding site.[5] Based on the structure of this enzyme, we have previously developed a strategy for generating catalytic antibodies with GSH binding sites and GPX activity using monoclonal antibody preparation technology and chemical mutation.[6] This study shows that the binding site for substrate GSH and the catalytic moiety selenocysteine are critical factors for catalysis.

Here we describe a new strategy for generating a GPX mimic with a GSH binding site using bioimprinting and chemical mutation. The method consists of five basic steps: (i) unfolding the conformation of the starting protein under acid conditions; (ii) addition of the imprinting molecule (or template), to allow it to interact with the denatured protein to form a new conformation with substrate binding sites; (iii) crosslinking the protein with a bifunctional reagent to fix the new conformation of the protein; (iv) dialysis of the protein to remove the imprinting molecules; and (v) conversion of the active serine residues in the binding site of the crosslinked protein into selenocysteines to incorporate catalytic groups. Thus, there are both substrate binding sites and catalytic groups in the printed protein and thus it should display an enzyme activity.

We chose a GSH derivative, *N*,*S*-bis(2,4-dinitrophenyl)glutathione (GSH-2DNP), as the imprinting molecule. The unstable thiol group and amino group of GSH were protected by reaction with 2,4-dinitrofluorobenzene to give GSH-2DNP. The derivative is structurally similar to the substrate molecule GSH. After the conformation of the starting protein (egg albumin) had been unfolded by denaturation, the imprinting molecule (GSH-2DNP) was allowed to interact with the unfolded protein to form a new conformation *via* hydrogen bonds, ion pairing and hydrophobic interactions. The new conformation was then fixed using glutaraldehyde. The cavities, which were left in the printed protein after removing GSH-2DNP by dialysis, were able to recognize the substrate GSH. The serine residues located in the binding sites of the imprinted protein were active and could be converted into the catalytic groups of GPX (selenocysteines) by chemical mutation.[6,7]

The preparation of the imprinted enzyme is as follows: egg albumin (1 ml of a 1 mg ml^{-1} solution) was adjusted to pH 3.0 with 0.1 M HCl and stirred at room temperature for 1 h. The imprinting molecule, GSH-2DNP (5 mg), was added to the protein solution and stirred for 1 h. The pH was then adjusted to 8.0 with 0.1 M NaOH and glutaraldehyde solution (1%; 100 μl) was added. The reaction mixture was stirred at 5 °C for 12 h and dialyzed for 48 h against 50 mM phosphate buffer, pH 7.0. The dialyzed solution was activated by adding 10 μL of phenylmethanesulfonyl flouride (20 mg ml^{-1} solution in MeCN) and incubated at 25 °C for 3 h. A portion of 1 M NaHSe (100 μl; 100 μmol) prepared according to the method of Klayman and Griffin[8] was added to the activated solution, and incubated at 40 °C for 30 h under nitrogen atmosphere. The solution was oxidized by air at 4 °C for 5 h and was centrifuged to remove the solid selenium. The crude imprinted protein was purified by gel filtration on a superose B-12 column, and the first peak was collected; the crosslinked protein in this peak proved to be the dimer by HPLC. The non-printed protein was also prepared from egg albumin using the same conditions in the absence of GSH-2DNP, and used as a control.



GSH-2DNP

The selenium content was determined by 5,5′-dithiobis(2-nitrobenzoic acid) titration,[7] and 1.89 ± 0.15 equiv. of selenium

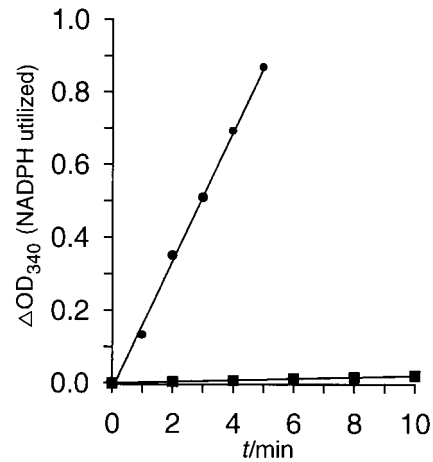


Fig. 1 Printed and non-printed protein-catalyzed reduction of H_2O_2 by GSH: (●) printed protein and (■) non-printed protein. The catalytic reaction containing GSH (1 mM), H_2O_2 (0.5 mM), glutathione reductase (1 unit), NADPH (0.25 mM) and 0.2 μM enzyme mimic (pH 7.0 at 25 °C) was monitored *via* NADPH decrease in absorbance at 340 nm. The absorbance change was corrected for the background reaction between H_2O_2 and GSH.

per printed protein molecule was detected. The GPX activity of the printed protein was measured by a coupled enzyme system containing glutathione reductase, GSH, NADPH and H_2O_2.[6] The printed protein-catalyzed reduction of H_2O_2 by GSH was initiated by adding the substrate H_2O_2. The initial rate of the printed protein (0.2 μM)-catalyzed removal of H_2O_2 (with NADPH) by GSH was found to be 8.2×10^{-3} M min^{-1} during the initial 5 min of reaction (Fig. 1). However, the initial rate of the non-printed protein (0.2 μM)-catalyzed reaction was only 9.5×10^{-5} M min^{-1}. The GPX activities of the printed and non-printed proteins were calculated to be 817 ± 28 and 10 ± 3 U μmol^{-1}, respectively. The printed protein displays a high GPX-like activity and is only seven times less than that of the native GPX from rabbit liver (5780 U μmol^{-1}).[6] Compared with ebselen (PZ51, 0.99 U μmol^{-1}), the best GPX mimic known,[6] the GPX activity of the printed protein is 820 times that for PZ51. The apparent catalytic rate constant for H_2O_2 (k_{cat}) was measured to be 785 min^{-1} ([GSH] = 1 mM).

It was found that GSH-2DNP inhibited the GPX activity of the printed protein, while the GPX activity of the non-printed protein was unaffected by GSH-2DNP, indicating that specific binding sites for the imprinting molecule have been obtained.

Thus, bioimprinting of albumin with a GSH derivative led to the generation of cavities for binding GSH. The high catalytic activity of the printed protein and the inhibition by the imprinting molecule provide strong evidence that the catalysis achieved is a function of specific binding sites generated by protein imprinting. Although the formation of active sites requires further investigation, the initial results suggest that this approach is a new method for generating imprinted enzymes with high GPX activity.

Notes and references

1 G. Wulff, *Angew. Chem., Int. Ed. Engl.*, 1995, **34**, 1812.
2 R. A. Lerner, S. J. Benkovic and P. G. Schultz, *Science*, 1991, **252**, 659.
3 S. Saraswathi and M. H. Keyes, *Enzyme Microb. Technol.*, 1984, **6**, 98.
4 S. C. Gamble, *J. Chem. Technol. Biotechnol.*, 1997, **68**, 123.
5 O. Epp, R. Ladenstein and A. Wendel, *Eur. J. Biochem.*, 1983, **133**, 51.
6 G. M. Luo, Z. Q. Zhu, L. Ding, Q. A. Sun, Z. Liu and J. C. Shen, *Biochem. Biophys. Res. Commun.*, 1994, **198**, 1240.
7 I. M. Bell, M. L. Fisher, Z. P. Wu and D. Hilvert, *Biochemistry*, 1993, **32**, 3754.
8 D. L. Klayman and T. S. Griffin, *J. Am. Chem. Soc.*, 1973, **95**, 197.

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