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An Fv catalytic antibody with high glutathione peroxidase activity

Jun-qiu Liu [a], Cheng-bo Shi [a], Gui-min Luo [a,*], Zi Liu [b], Guo-li Zhang [b], Weiying Ma [b], Jia-cong Shen [c]

[a] *The National Laboratory of Enzyme Engineering, Jilin University, Changchun 130023, China*
[b] *Changchun University of Agriculture and Animal Sciences, Changchun 130061, China*
[c] *Key Laboratory for Supramolecular Structure and Spectroscopy of Jilin University, Changchun 130023, China*

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Abstract

An Fv catalytic antibody with high glutathione peroxidase (GPX) activity was prepared using proteolysis of a monoclonal antibody 3H4 (IgM), and subsequent chemical mutation. The Fv fragment generated by pepsin digestion of 3H4 retained binding activity for glutathione (GSH), one substrate of GPX. Active serines in the binding site of the Fv fragment were converted to selenocysteine, the catalytic group of GPX. The selenium-containing Fv fragment exhibits a high GPX activity of the same order of magnitude as native GPX from rabbit liver. © 1999 Elsevier Science S.A. All rights reserved.

Keywords: Fv; Catalytic antibody; Glutathione peroxidase; Selenium

1. Introduction

The immune system provides a means to generate high affinity antibodies for antigens [1]. When stable transition state analogues of organic intermediate are used as immunogens, catalytic antibodies can be generated using monoclonal technology [2]. In recent years, many catalytic antibodies catalyzing a wide variety kinds of reactions have been prepared using this method [3,4]. In our previous studies, we developed a new strategy for generating catalytic antibodies with a high GPX activity that reached or exceeded that for native GPX activity [5,6]. This strategy requires generation of a binding site with a high affinity for the substrate, glutathione (GSH), by monoclonal technology and subsequent incorporation of a catalytic moiety, selenocysteine, into the binding site by chemical mutation. Glutathione peroxidase (GPX, 1.11.1.9) is an important antioxidative enzyme [7]. It catalyzes the reduction of a wide number of hydroperoxides (ROOH) with GSH, preventing biomembranes from oxidative damage, thereby possessing potential to treat or study certain cardiovascular, tumorous and endemic disease states [8].

Although the catalytic antibodies we previously prepared have high GPX activity, these remain unsuitable for clinical application because of their high immunogenicity, high molecular weight and poor availability. The Fv fragment of an antibody is the minimal antigen-binding region. The therapeutic efficacy of Fv fragments hinges on antigen recognition, weak immunogenicity and good delivery [9]. In this work, we prepared Fv fragments from the 3H4 IgM mouse antibody and created the Fv catalytic antibody. The catalytic properties of this new Fv catalytic antibody have been studied.

2. Materials and methods

2.1. Materials

Mouse McAb 3H4 (IgM) was prepared as previously described [5] and stored at −20°C. Pepsin, sodium boro-

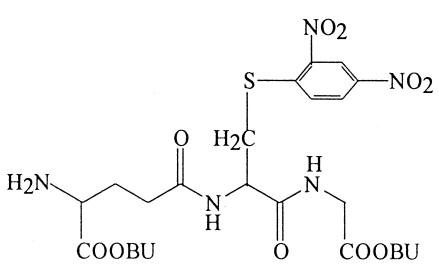

Fig. 1. Structure of hapten.

* Corresponding author. E-mail: gmluo:mail.jlu.edu.cn

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Table 1
Comparison of some properties of McAb 3H4 and Fv
The concentration of protein was determined by Lowry method.

	Molecular weight (kDa)	GSH binding sites	GSH association constant (M^{-1})
Fv	2.6	1	$(1.1 \pm 0.3) \times 10^7$
McAb 3H4	–	10	$(8.6 \pm 0.5) \times 10^6$

hydride, GSH, NADPH, phenylmethane sulfonyl fluoride (PMSF) and glutathione reductase (Type III) were obtained from Sigma. NaHSe was prepared according to the method of Klayman and Griffin [10].

2.2. Preparation of the Fv fragment from mouse McAb 3H4 (IgM)

The mouse McAb 3H4 was digested with pepsin (1:100 ratio of pepsin to 3H4) for 1 h in a phosphate buffer, pH 3.8, at 37°C. Fv was then isolated from this solution by gel filtration on Superose B12 (Pharmacia) with a yield of 50% (Fig. 1). The Fv was freeze-dried and stored at −20°C.

2.3. Determination of Fv molecular weight

The molecular mass of the purified Fv was determined by HPLC (Waters) gel filtration on a Protein Pak-125 column, 5 mM phosphate buffer was used as the mobile phase.

2.4. Binding activity of Fv for GSH

The binding activities of Fv and 3H4 to GSH were performed by titration of fluorescence quenching [11]. Fluorescence measurements were made by recording the absorbance with a Hitachi 850 spectrophotometer (excitation, 280 nm; emission, 342 nm).

2.5. Preparation of the Fv catalytic antibody

0.5 mg of Fv was dissolved in 0.5 ml of 100 mM potassium phosphate, pH 7.0, PMSF 10 μl solution (20 mg/ml in acetonitrile) was added and incubated for 1 h at room temperature. The sulfonylated Fv was then treated with 20 μl of 1 M NaHSe for 40 h at 40°C. The crude reaction mixture was purified first by centrifugation, then using Bio-gel P-10 column and was finally freeze-dried.

2.6. Determination of Fv selenium content

Selenium content of the Fv derivative was measured by the DTNB method [12]. Fv was treated with excess sodium borohydride in PBS buffer under nitrogen atmosphere for 30 min, the solution was treated with 1 M imidazole to quench the excessive reducing agent and adjusted to pH 7.0 with 1 M HCl, and 100 μl of the Fv enzyme was added to 5 ml of DTNB solution (0.1 mM). The concentration of selenol in enzyme was determined from the absorption of 3-carboxy-4-nitrobenzenethiolate at 410 nm (ε = 11 400 M^{-1} cm^{-1}, pH 7.0).

Table 2
GPX activity of mutated Fv, mutated 3H4, PZ51 and the native GPX from rabbit liver

Enzyme	Activity (U/μmol)
Ebselen (PZ51)	0.99[16]
Mutated Fv	2500
Mutated 3H4	12 000
Native GPX	5780[5]

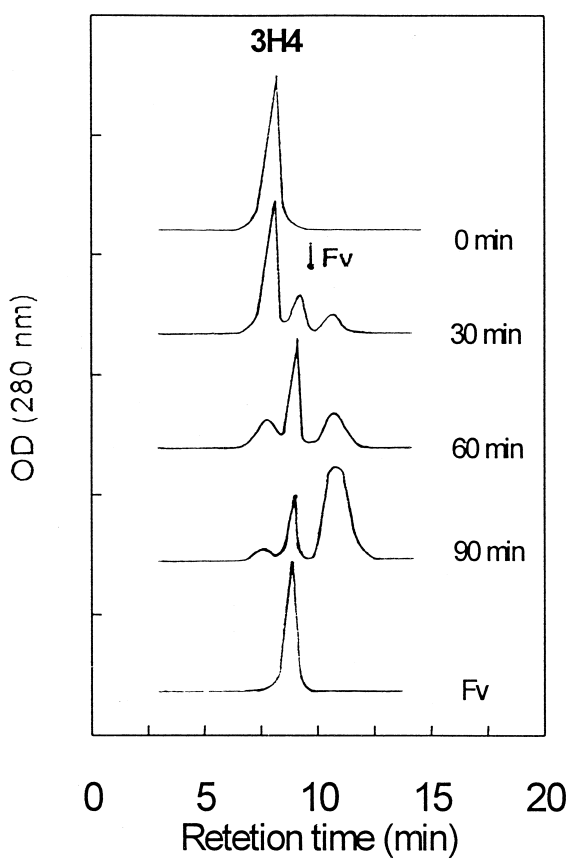

Fig. 2. HPLC elution patterns of 3H4 digested by pepsin on a protein Pak-125 column at different times. The digestion conditions are described in Section 2. The column was equilibrated and eluted with 50 mM phosphate buffer, pH 7.0.

2.7. Determination of GPX activity

GPX activity was determined by the method of Wendel [13]. The reaction was carried out at 37°C in 700 μl of solution containing 50 mM pH 7.0, potassium phosphate buffer, 1 mM EDTA, 1 mM sodium azide, 1 mM GSH, 0.25 mM NADPH, 1 unit of GSH reductase, 10–100 μM of Fv. The reaction was initiated by addition of 0.5 mM H_2O_2. GPX activity was determined by the decrease in NADPH absorption at 340 nm. Appropriate controls were run without enzyme and were subtracted. The activity unit of the enzyme is defined as the amount of enzyme that utilizes 1 μmol of NADPH per minute. The activity is expressed in U/μmol of enzyme.

3. Results and discussion

In the study of catalytic antibody, hapten design is one of the most critical factors. According to the known structure and mechanism of native GPX, the GPX binding site is specific for substrate GSH, the active site selenium of GPX alternates between reduced and oxidized forms in the reaction procedure (Eqs. (1)–(3)) [14].

$$\mathrm{ROOH + ESeH} \xrightarrow{k_1} \mathrm{ESeOH + ROH} \quad (1)$$

$$\mathrm{ESeOH + GSH} \xrightarrow{k_2} \mathrm{ESeSG + H_2O} \quad (2)$$

$$\mathrm{ESeSG + GSH} \xrightarrow{k_3} \mathrm{ESeH + GSSG} \quad (3)$$

This bimolecular reaction was demonstrated to be very complicated and proceeds through several intermediates. While fully considering the major factors including antibody binding site with specific affinity for substrate GSH and a selenium prosthetic group, we designed a hapten, GSH–DNP–dibutyl ester (Fig. 1). Using a monoclonal technique, we perviously prepared McAb 3H4 generated against this hapten. 3H4 displayed a high affinity for GSH (Table 1). After chemical mutation, the GPX activity of 3H4 catalytic antibody exceeds the native GPX activity (see Table 2). This result indicates that the recognition of antibody 3H4 for GSH plays an important role for catalysis. This recognition is retained in Fv fragments as shown in Table 1.

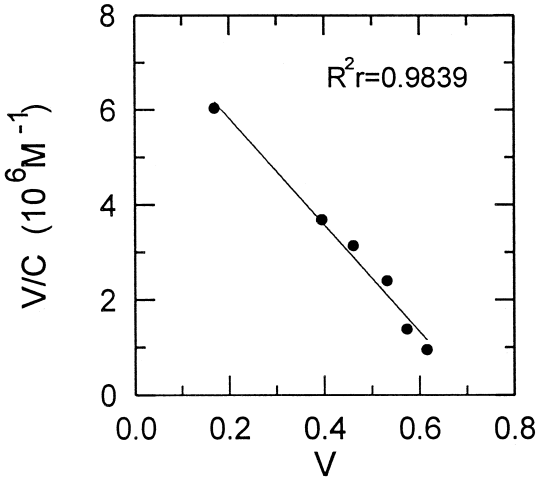

Fig. 4. Scatchard plot analysis of the binding of substrate GSH to the binding site of Fv, where V is the number of GSH molecules bound per binding site, and C is the concentration of GSH.

3.1. Preparation of the Fv fragment

Preparation of Fv from IgA was developed by Hochman et al. [15]. This method can be used for digestion of IgM as well. The digestion of McAb 3H4 with pepsin was

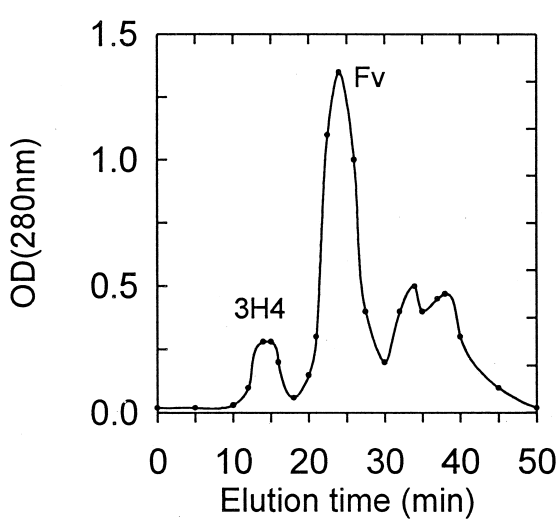

Fig. 3. Purification of Fv from digestion solution of 3H4 on Superose B12. The column was equilibrated and eluted with 50 mM phosphate buffer, pH 7.0.

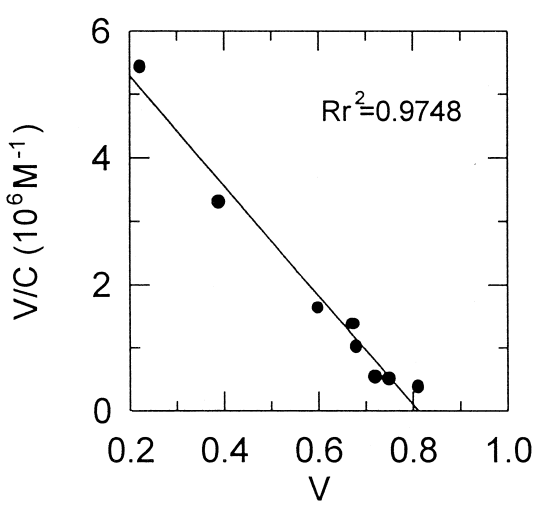

Fig. 5. Scatchard plot analysis of the binding of GSH to the binding site of McAb 3H4, where V is the number of GSH molecules bound per binding site, and C is the concentration of GSH.

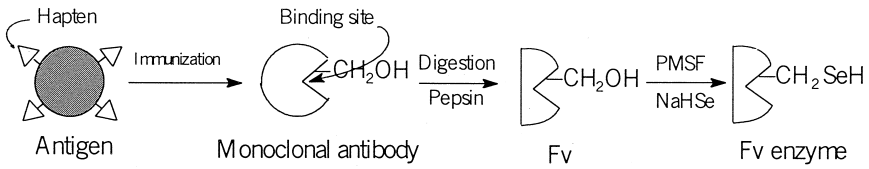

Fig. 6. Preparation of Fv catalytic antibody from monoclonal methods.

followed by HPLC (Fig. 2). When 3H4 was digested at pH 4.5 for 6 h, no Fv fragments were observed. However, a good yield (50%) of Fv can be obtained by digesting 3H4 with pepsin at pH 3.8 for 1 h. After 1 h of digestion, however, the Fv can be degraded into small fragments.

The molecular mass of the Fv fragment product was measured by HPLC. It was found to be 26 kDa and was identical to that of an Fv fragment (Fig. 3) [9].

3.2. Binding activity of Fv for GSH

The antibody Fv fragment is the smallest functional fragment maintaining a high affinity binding for antigen. High affinity binding for GSH by the Fv fragment is a key factor for catalysis. The Fv binding constant for GSH was determined by titration of fluorescence quenching. Values were then calculated from Scatchard plots to be 1.1×10^7 M^{-1} (see Fig. 4 and Table 1). Affinity of Fv for GSH is similar to that for McAb 3H4 (see Fig. 5 and Table 1). The results show that the Fv fragment retains a binding site as well as the conformation of variable regions present in McAb 3H4.

3.3. Preparation and catalytic activity of the Fv catalytic antibody

The entire preparation procedure for the antibody Fv catalytic antibody is shown schematically in Fig. 6. Active serine residues in the antibody variable regions can be converted to selenocysteines by chemical mutation [5,12]. Therefore, the essential catalytic selenocysteines can also be incorporated into the GSH binding site of Fv. The method for preparing the Fv enzyme is the same as that reported for preparing McAb 3H4 enzyme. The selenocysteine content of the mutated Fv product was measured by DTNB method [12] and found to be 2.20 mol/mol Fv molecule. The GPX activity of the mutated Fv was measured using a coupled enzyme system. Catalytic reduction of H_2O_2 by GSH was found to be 2500 U/μmol, which is approximately of the same order of magnitude as that activity for native GPX from rabbit liver and is 2500 times more than that of PZ51, the best GPX mimic with low molecular weight known in the world (Table 2) [16].

The experimental data show that the antibody-derived Fv fragment containing a GSH binding site can be successfully generated by pepsin digestion. This strategy is relatively simple compared with the preparation of a single chain Fv (ScFv) by protein engineering. Using this strategy and chemical mutation, an Fv catalytic antibody with a high GPX activity was obtained. Because of its high GPX activity, low molecular mass and potential for reduced immunogenicity, we anticipate that it will be applied in medicine for treatment of a number of pathologies.

Acknowledgements

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