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Human catalytic antibodies with glutathione peroxidase activity

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Abstract

In order to generate catalytic antibodies with glutathione peroxidase (GPx) activity, we prepared GSH-S-DNP butyl ester and GSH-S-DNP benzyl ester as the haptens. Two ScFvs that bound specifically to the haptens were selected from the human phage-displayed antibody library. The two ScFv genes were highly homologous, consisting of 786 bps and belonging to the same VH family-DP25. In the premise of maintaining the amino acid sequence, mutated plasmids were constructed by use of the mutated primers in PCR, and they were over-expressed in *E. coli*. After the active site serine was converted into selenocysteine with the chemical modifying method, we obtained two human catalytic antibodies with GPx activity of 72.2U/μmol and 28.8U/μmol, respectively. With the aid of computer mimicking, it can be assumed that the antibodies can form dimers and the mutated selenocysteine residue is located in the binding site. Furthermore, the same Ping-Pong mechanism as the natural GPx was observed when the kinetic behavior of the antibody with the higher activity was studied.

Keywords: Glutathione peroxidase (GPx); Human catalytic antibody

1. Introduction

Natural glutathione peroxidases are a series of anti-oxidant enzymes, with the capacity of binding to thiol, and some of these are specific for glutathione. As a kind of biosynthetic selenoenzyme [1], the selenocysteine (Sec) residue is indispensable to its catalyzing the reduction of hydrogen peroxide and other hydroperoxides both in normal and diseased cells, ultimately reducing the lesion of biomembranes. Recently, GPx has also been reported to provide protection against viral-induced myocarditis [2] and to be correlative with HIV disease progression [3]. However, natural GPx is unstable and the half-life in human body is short [4], so its sources and application were restricted. On the other hand, the biosynthesis of selenocysteine required both the UGA codon, which can be translated into Sec and the selenocysteine insertion sequence (SECIS), and the expression of natural GPx in *E. coli* is restricted because SECIS is very different in eucaryote and procaryotic [5]. Due to the important biological function of GPx and the increasing interest in the biochemistry of selenium, different selenoorganic mimics have been synthesized and studied [6].

The technology of catalytic antibodies was developed since 1986, and previous investigations has verified that catalytic antibodies can be used in medicine [7]. For example, catalytic antibodies have been used to hydrolyze cocaine to be a nontoxic state [8] and to be prodrugs for target therapy [9]. This method, together with chemical modifying method, has been recently used by Ding to synthesize a monoclonal antibody with GPx activity higher than some natural GPxs [10,11]. In the antibody selected, there is no GPx activity before the introduction of selenium. However, the GPx activity increased by several folds after the serine residue was converted into selenocysteine with chemical modification.

Because of the inherent immunogenicity of murine antibody [12], the application of catalytic antibodies derived from monoclonal antibodies is limited in clinical therapy. It has been reported that by shuffling genomic exons, simple chimeric antibodies with murine variable regions and human constant regions have been made [13]. Such chimeric antibodies are superior to murine isotypes in that they could reduce the anti-globulin responses during the therapy.

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The construction of human combinatorial library of the antibody repertoire is another important way to generate human immunoglobulins [14]. Using the bacteriophage lamda vector system which can be expressed in *E. coli*, specific antibodies can be screened rapidly. Importantly, the immunogenicity of the human derived catalytic antibodies is very low. Here we report the successful preparation of human catalytic antibodies with GPx activity from a human antibody library.

2. Experimental procedures

2.1. Materials

The phage-displayed antibody library was kindly supplied by Dr. Winter, G. Plasmid pET21a was purchased from Novagen, and pCANTAB 5E was purchased from Pharmacia. Restriction endonucleases were purchased from New England Biolab. Bacterial strains used were *E. coli* TG1, BL21 (DE3), and HB2151. GSH, PMSF, NADPH were purchased from Sigma. Glutathione reductase was purchased from Boehringer-Mannheim GmbH. Monoclonal antibody (mAb) anti-E-tag and 9E10 were purchased from Pharmacia. Sephadex G-75, G-10 came from Pharmacia.

2.2. Synthesis of haptens

In according to the strategy described by Ding [10,11], GSH-S-DNP Butyl Ester and GSH-S-DNP Benzyl Ester was designed as hapten3 (Hp3) and hapten4 (Hp4), and the haptens were synthesized by the method reported [15,16] and characterized by infrared spectroscopy (IR), NMR and mass spectroscopy (MS).

2.3. Rescue and selection of phage library

The phagemid particles were rescued from the phage library as described [17]. The library cells at a density of $OD_{600}=0.5$ were infected with M13K07 helper phage, and the mixture was incubated for 30 min at 37°C. The phage particles were rescued from the culture and concentrated. Phages were selected for three rounds as described previously [18], and the specificity of the selected antibody was checked with ELISA.

2.4. Screening and sequencing of clones

Soluble ScFv fragment was secreted from a non-suppressor strain of bacteria HB2151, which were infected by the selected phage. The specificity of ScFvs to antigen was assayed by ELISA via the use of a mouse monoclonal antibody (mAb) 9E10, which recognizes the c-myc peptide tag, and HRP-goat anti-mouse IgG was used as the secondary antibody. Western blot analysis was performed to detect the binding activity of soluble ScFv that had come from the culture supernatants and periplasmic extracts. The concentrated culture supernatants and periplasmic extracts (which include 2 μg ScFv) were run on 12% SDS–PAGE and then electroblotted. The filters were blocked by 5% MPBS for 2 h at room temperature and then mAb 9E10 was added and incubated overnight at 4°C. After washing with PBST (PBS containing 0.2% Tween 20) and PBS, HRP-goat anti-mouse IgG was used to detect the bound 9E10. The clone which gave the strongest positive ELISA signal was sequenced by use of dideoxy method [19].

2.5. Computer-assisted structural analysis

We used the overall backbone template of an immunolgobulin, anti-phosphatidylinositol specific phospholipase C diabody (PDB code: 1LMK), as the template of the protein. The other homologous templates 1 VGE (chain H), 1ADQ (chain L) and 1NFD (chain B) were also used for refined homology modeling. The program RASMOL was used to mimic the structure. The coordinates of the linker (residues 123–137) were not included, because their conformations are difficult to determine and less important to the function.

2.6. Construction and over-expression of human ScFv

The construction was made in the standard ways [20]. The SfiI-NotI fragment containing the ScFv gene was cut off from pHEN1, on which the library was built, and was ligated into pCANTAB 5E encoding E-tag, which made the detection and purification of the ScFv simple. Then PCR was performed with recombinant pCANTAB 5E as the template. The amplified product was ligated to pET21a with the cut at NdeI and HindIII restriction sites, which generated the human ScFv over-expression recombination vector. To increase the yield of antibody expression, on the premise of maintaining the amino acid sequence, two forward primers and one reverse primer were designed in PCR. One primer corresponded to the encoding gene (5′-AGATATACATATGGCCCAGGTG), and the other one had six mutated sites (5′-GATATACATATGGCTC-AGGTTCAGCTGGTTCAATCAGGT), both the two forward primers containing NdeI restriction site. The back primer (5′-AGCAAGCTTTTATTATGCGGCACGCGG) that overlapped part of the 3′ end of the coding sequence contains a HindIII site.

The recombinant was transformed into *E. coli* BL21 (DE3). As the density of cells reached $OD_{600}=0.3–0.9$, the expression was induced with 4 mM IPTG (isopropyl-β-D-thiogalactopyranoside), growth was continued for 4–5 h at 37°C.

2.7. Refolding and purification of the ScFv

The cells harvested (1.5 g) from a 1 liter culture were resuspended in 50 mM Tris–HCl (pH=8.0, containing 10 mM β-mercaptoethonal and 0.01% lysozyme). The mixture was then sonicated for 1.5 min with intervals of 15 s. After centrifugation (12 000 rpm, 30 min) and washing with 2 M NaCl, 0.5% Triton X-100 and 4 M urea, inclusion body (0.3 g) was recovered and dissolved in 10 ml denaturing buffer (6 M guanidine hydrochloride/50 mM Tris, pH= 8.0). The resulting solution was filtered through 0.45 μm membrane and then centrifuged (12 000 rpm, 30 min). The supernatant was saved and the protein concentration of the denatured inclusion bodies was determined with the BIO-RAD Protein Assay Kit.

In the process of refolding the protein, a dilute solution of denatured inclusion bodies was rapidly placed into 50 ml of refolding buffer (0.4 M Tris–HCl/0.5 M Arg/2 mM EDTA/2 M guanidine hydrochloride, pH=8.0). Every 1 h, denatured solution was added to the refolding buffer, which was stored unstirred at 4°C, increasing the protein concentration by 10 μg/ml. After five cycles, a final protein concentration of 50 μg/ml was reached. The refolding buffer was incubated overnight, then was concentrated on a membrane (10 KD molecular weight-cut off) by several dilution to remove the guanidine hydrochloride. ELISA was used to assay the binding activity of refolded protein. The concentrated refolded protein was loaded onto an affinity anti E-tag column and subsequently onto Sephadex G-75 column.

2.8. Introduction of selenium and assay of the enzyme activity

Sodium hydrogen selenide was prepared according to the method described by Klayman and Griffin [21]. The protocol of conversion of the active site serine into selenocysteine with PMSF was improved by Wu and Hilvert [22]. After gel filtration of the crude reaction mixture on Sephadex G-10, the protein fraction Se-ScFv was collected. The GPx activity was assayed directly.

According to DTNB method described previously [23], the Sec content of modified antibody was calculated by measuring the total thiol groups of modified antibody. The dissociation constants for both ScFv and Se-ScFv with haptens were measured by ELISA.

The enzyme activity assay was carried out as described by Wendel et al. [24], using hydrogen peroxide as the substrate in the presence of GSH with NADPH as a cofactor. The decrease in NADPH concentration spectrophotometrically monitored at 366 nm is a measure of GPx activity.

2.9. Kinetic study

Kinetic parameters were determined by Dalziel analysis. To investigate the dependence of the rate on substrate concentration, the reaction rates were determined at several concentrations of H_2O_2 while keeping the concentration of GSH constant. Three groups of data were obtained in this way.

3. Results

The prepared hapten3 and hapten4 were characterized by NMR, IR and MS, with the results showing that the products were the designed haptens.

After three rounds of panning and ELISA detection, five of 55 and five of 50 specific ScFv antibodies displayed on phage against Hp3 and Hp4, respectively, were obtained. The specific antibodies that gave the strongest ELISA signal, were named 3B10 to Hp3, and 4F4 to Hp4. The result of ELISA and Western blot analyses showed that soluble 3B10 and 4F4 were able to recognize and bind the respective hapten and they could be found in culture supernatant and periplasmic extracts (Fig. 1). The molecular weights of both 3B10 and 4F4 were 31 kD.

The nucleotide sequence of the VH segments of 3B10 and 4F4 (Fig. 2) were compared with the germline gene segments presented on the VH directory compiled by the library constructor [14] (Table 1). It was found the ScFv gene consisted of 786 bps, and belonged to the VH family-DP25. The tertiary structure of the protein was obtained by use of a computer model, which showed that the protein is most likely to function as a dimer and the amino acids in the CDR3 region can form a pocket which may be located on the surface of the protein (Fig. 3).

When expressing the protein with unmutated primers,

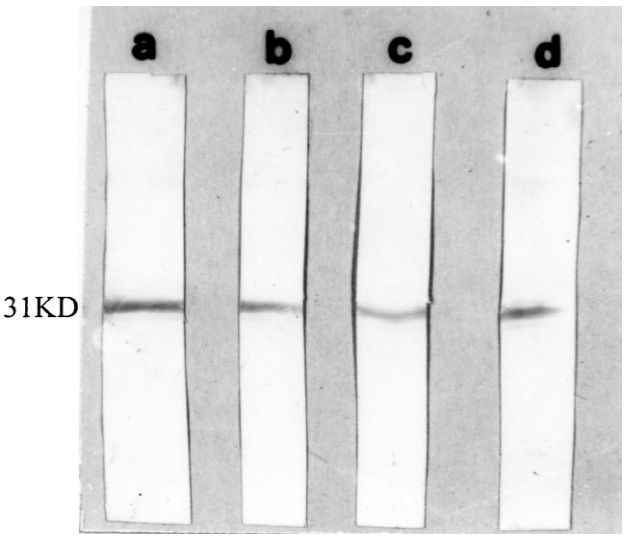

Fig. 1. Western blot analysis of soluble scFvs. (a) Concentrated *E. coli* cell expressing 3B10 culture supernatant. (b) Periplasmic extract from *E. coli* cell expressing 3B10. (c) Periplasmic extract from *E. coli* cell expressing 4F4. (d) Concentrated *E. coli* cell expressing 4F4 culture supernatant.

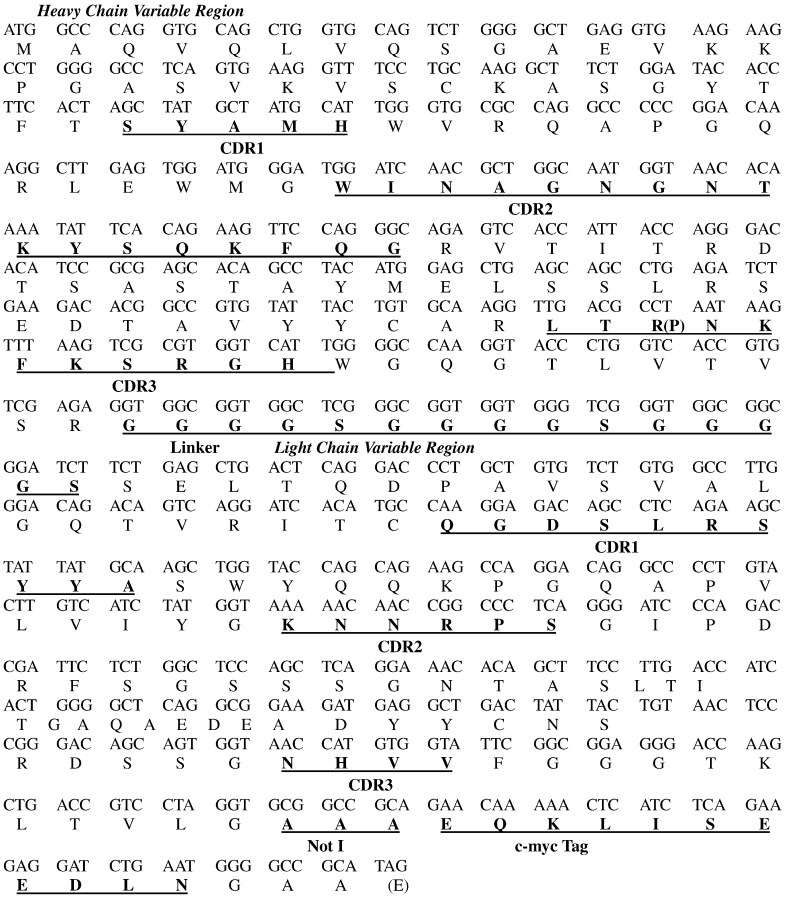

Fig. 2. The nucleotide sequence of 3B10 (103 is R) and 4F4 (103 is P).

the yield is very low, while with the mutated primers the yield is much higher. By use of the mutated primers, over-expression 3B10 and 4F4 were achieved. Western blot analysis confirmed that the highly expressed foreign protein was our desired ScFv (Fig. 4), exhibiting the expected molecular weight. The amount of expressed desired proteins was approximately 100 mg per liter culture. The results of SDS–PAGE indicated that the ratio of expressed antibody to total *E. coli* protein was approximately 40%. Moreover, the results of SDS–PAGE indicated that the recovered 3B10 and 4F4 show two bands. The molecular weight of the more prominent band was 31 kD. The molecular weight of the other band was about 62 kD.

The products we obtained had no GPx activity without selenium. After introducing selenium into the 3B10 and 4F4, we found they exhibited GPx activity of 72.2U/μmol and 28.8U/μmol protein respectively, while the binding to the haptens changed little. The DTNB results show there is about one Sec residue in every protein molecule in the mutated antibody. Three parallel lines of Dalziel plots were constructed by holding three different GSH concentrations constant while varying the concentration of H_2O_2 respectively, which is consistent with the Ping-Pong mechanism involved in the natural GPx [25] (Fig. 5).

Table 1
Deduced VH-CDR3 sequences and germline origin of both 3B10 and 4F4

Hapten	scFv	CDR3 sequence	Family	Segment
3	3B10	LTRNKFKSRGH	VH1	DP25
4	4F4	LTPNKFKSRGH	VH1	DP25

4. Discussion

Theoretically, catalytic antibodies can catalyze chemical transformations whether a natural enzyme to the reaction exists or not. When a target is selected, a catalytic antibody is always there. As a new class of potential therapeutic agents, catalytic antibodies might mediate the selective destruction of viral coat proteins or tumor antigens and may prove efficacious in the lysis of blood clot. Thus, catalytic antibodies provide a novel path for drug designers. Unlike the catalytic antibodies that catalyze the organic reaction, monoclonal antibodies obtained from immunized mice will cause Human Anti-Mouse Antibody (HAMA) when they were used in humans, so they cannot be used directly. On the other hand, human catalytic antibodies should be more advantageous in clinical

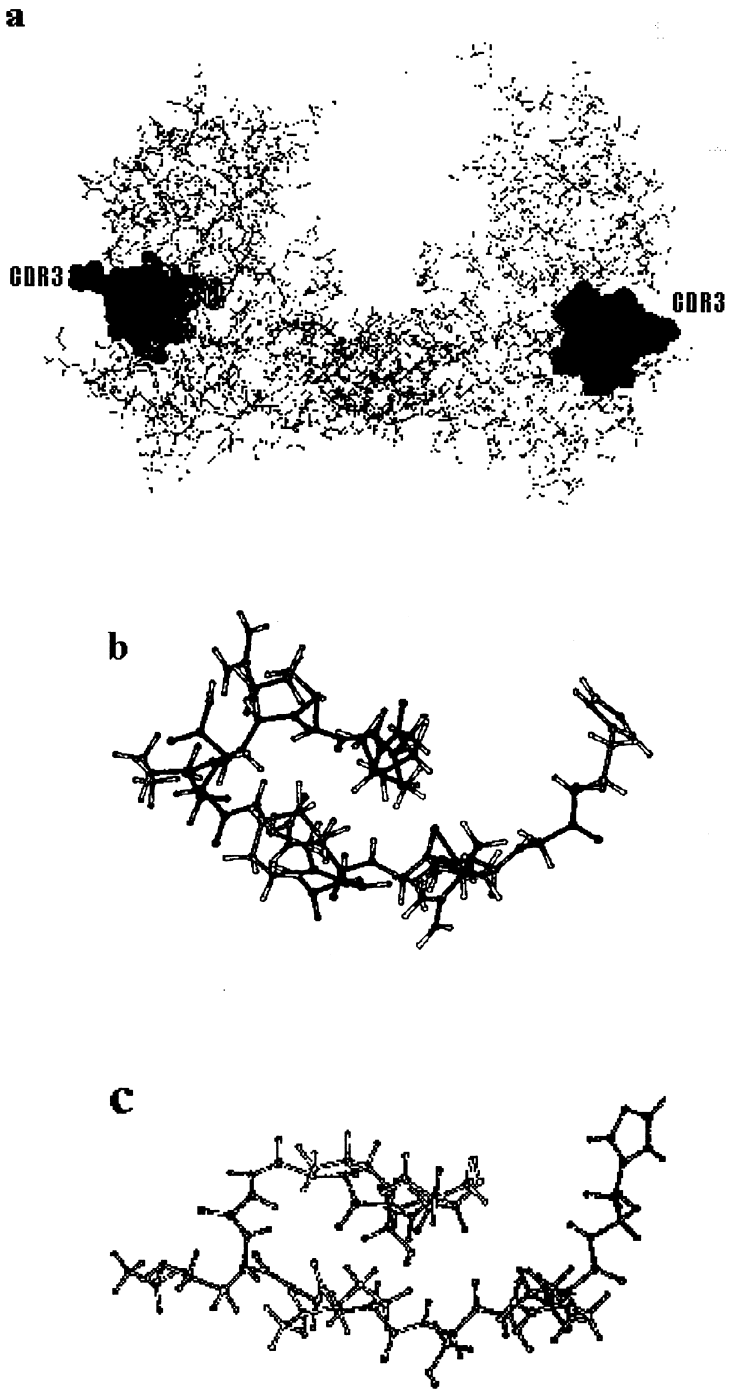

Fig. 3. Model structure by use of Rasmol analyzing software. (a) Dimer of ScFv 3B10, (b) CDR3 region of 3B10, (c) CDR3 region of 4F4.

therapy, but so far there have been few reports on human catalytic antibodies [26].

The phage-displayed antibody library has been verified to be a powerful means to create novel catalytic antibodies of human sources. A variety of catalytic antibodies can be derived from an antibody library with the variety and specificity depending on the size of the library. The library used in this work contained 10^7 different clones, and we have obtained several different clones specific to Hp3 and Hp4 from this library. The antibodies we expressed in the research were those that bound the haptens with the greatest affinity. Since the catalytic activity has no relationship to binding capacity [27], we will express additional antibodies in hope to produce catalytic antibodies with higher activity.

Here we have prepared the human catalytic antibodies with GPx activity by the chemical modification of specific antibodies obtained from a human phage-displayed anti-

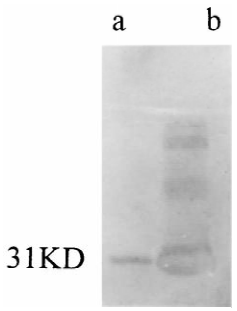

Fig. 4. Western blotting of high level expressed 3B10. (a) Purified renatured 3B10 on Sephadex G-75, (b) *E. coli* cell proteins after induction.

body library. The negative control protein with Sec by chemical modifying method has been proved to have GPx activity of only 10U/μmol [28], so the GPx activity of our catalytic antibodies is due to their specific binding to the haptens. Although their activities are somewhat low comparing to the activity of the natural rabbit liver GPx, which is 5780U/μmol, they have many advantages to be used. The highly expressed catalytic antibodies Se-3B10 and Se-4F4 should express lower immunogenicity when they are used in therapy. Furthermore, their smaller size should make them easier to be absorbed. Because they are easier to crystallize than natural GPx, their structures and reaction mechanism are more amenable to study.

As seen from the results of the gene sequencing, the sequences of CDR3 region of the ScFv and GSH binding site of native GPx do not resemble. The main reason is that they belong to the different families, antibody and enzyme. However, according to the data of binding and catalyzing, we may suppose their tertiary structure are similar, which contribute to the catalytic activity of the antibodies. On the other hand, it can be expected that if we made some mutations on the CDR3 region according to the GSH binding site of native GPx, the mutated antibodies will have higher activity than 3B10 and 4F4.

Our many results indicate that ScFv 3B10 and 4F4 can form dimers after purification. After refolding the ScFv fragment emerged in two peaks, corresponding in size to a monomer and dimer on Sephadex G-75. The dimer could not be dissociated in presence of dithiothreitol, and was therefore not linked by disulphide bonds. The computer model also showed that the two subunit bind in the site of the linker. In fact, the mimicking should be successful, since the template 1LMK is similar to 8FAB (also an Fv dimer) that was used in the paper by Hirose et al. [29], but it is probably better than 8FAB due to the closer topological relationship between ScFv and 1LMK. Another result in the computer model is that the CDR3 is on the surface. In the natural GPx, there is Arg-167 in the active region, which binds to GSH. In the most kinds of natural GPx, Arg, Gln, Ser, and Thr often bind to GSH by hydrogen bonds. The ScFv shows similar hydrogen bonding. This indicates that the reaction mechanism of the ScFv should be similar to the natural GPx. The results obtained from the kinetic analysis have proved it.

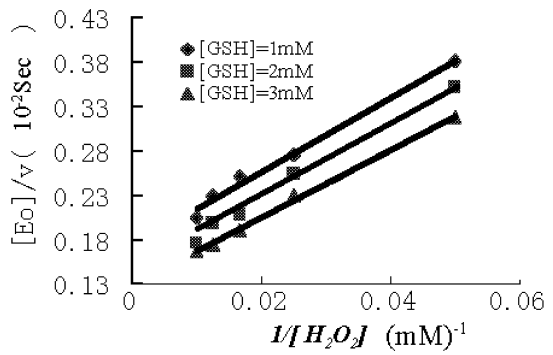

Fig. 5. Dalziel curve showed the mechanism of the Se-3B10 is Ping-Pong mechanism similar to the natural GPx.

In conclusion, a human antibody library is capable of providing various human antibodies for producing catalytic antibodies for clinical therapy, and in the future human catalytic antibodies with higher catalytic activity may emerge by affinity maturation of selected antibodies by chain shuffling. The use of this library has highlighted the new possibilities for the application of catalytic antibodies in medicine.

5. Notation

GSH, reduced glutathione; cGPx, cytosolic glutathione peroxidase; GSH-S-DNP, *S*-(2,4-dinitrophenyl) glutathione; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PMSF, phenylmethanesulfonyl fluoride

References

[1] J.W. Forstrom, J.J. Zakowski, A.L. Jappel, Biochemistry 17 (1978) 2639–2644.
[2] M.A. Beck, R.S. Esworthy, Y.S. Ho, F.F. Chu, FASEB J. 12 (1998) 1143–1149.
[3] M.K. Baum, P.G. Shor, Nutr. Foods AIDS (1998) 71–79.
[4] A. Wendel, P. Cikryt, FEBS Lett. 120 (1980) 209–211.
[5] M.J. Berry, L. Banu, Y. Chen, S.J. Mandel, J.D. Kieffer, J.W. Harney, P.R. Larsen, Nature 353 (1991) 273–276.
[6] S.R. Wilson, P.A. Zucker, R.C. Huang, A. Spector, J. Am. Chem. Soc. 111 (1989) 5936–5939.
[7] P. Wentworth, K.D. Janda, Curr. Opin. Chem. Biol. 9 (1998) 109–115.
[8] B. Mets, G. Winger, C. Cabrera, S. Seo, S. Jamdar, G. Yang, K. Zhao, R.J. Briscoe, R. Almonte, J.H. Woods, D.W. Landry, Proc. Natl. Acad. Sci. USA 95 (1998) 10176–10181.
[9] P. Wentworth, A. Datta, D. Blakey, T. Boyle, L.J. Partridge, G.M. Blackburn, Proc. Natl. Acad. Sci. USA 93 (1996) 799–803.
[10] Z.Q. Zhu, L. Ding, G.M. Luo, Z. Liu, Q. Sun, T.S. Yang, J.C. Shen, Biochem. Biophys. Res. Commun. 202 (1994) 1645–1650.
[11] L. Ding, Z. Liu, G.M. Luo, D.Q. Zhao, J.Z. Ni, Biochem. J. 332 (1998) 251–255.

[12] R.A. Miller, A.R. Oseroff, P.T. Stratte, R. Levy, Blood 62 (1983) 988–995.
[13] L. Riechmann, M. Clark, H. Waldmann, G. Winter, Nature 332 (1988) 323–327.
[14] I.M. Tomlinson, G. Walter, J.D. Marks, M.B. Leiwelyn, G. Winter, J. Mol. Biol. 227 (1992) 776–798.
[15] M.E. Anderson, F. Powrie, R.N. Puri, A. Meister, Arch. Biochem. Biophys. 239 (1985) 538–548.
[16] L. Ding, G.M. Luo, Z. Liu, D.Q. Zhao, J.Z. Ni, Sci. Sin. B. Chem. Biol. Agric. Med. Earth Sci. 27 (1997) 295–301.
[17] H.R. Hoogenboom, G. Winter, J. Mol. Biol. 227 (1992) 381–388.
[18] A. Nissim, H.R. Hoogenboom, I.M. Tomlinson, G. Flynn, C. Midhley, D. Lane, G. Winter, EMBO J. 13 (1994) 692–698.
[19] F. Sanger, S. Nicken, A.R. Coulson, Proc. Natl. Acid. Sci. USA 74 (1977) 5463–5467.
[20] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.
[21] D.L. Klayman, T.S. Griffin, J. Am. Chem. Soc. 95 (1973) 197–199.
[22] Z.P. Wu, D. Hilvert, J. Am. Chem. Soc. 111 (1989) 4513–4514.
[23] D. Cavallini, M.T. Graziani, S. Dupre, Nature 212 (1966) 294–295.
[24] A. Wendel, W. Piiz, R. Ladenstein, G. Sawatzki, U. Weser, Biochem. Biophys. Acta 377 (1975) 211–215.
[25] K. Dalziel, Biochem. J. 114 (1969) 547–556.
[26] M. Baca, T.S. Scanlan, R.C. Stephenson, J.A. Wells, Proc. Natl. Acad. Sci. USA 94 (1997) 10063–10068.
[27] H.D. Ulrich, P.G. Schultz, J. Mol. Biol. 275 (1998) 95–111.
[28] J. Liu, G.M. Luo, S. Gao, K. Zhang, X. Chen, J.C. Shen, Chem. Commun. (1999) 199–200.
[29] M. Hirose, T. Hayano, H. Shirai, H. Nakamura, M. Kikuchi, Protein Eng. 11 (1998) 243–248.