Expression of Fab fragment of catalytic antibody 6D9 in an *Escherichia coli* in vitro coupled transcription/translation system

XiuPing Jiang[a], Yuji Ookubo[b], Ikuo Fujii[b], Hideo Nakano[a,*], Tsuneo Yamane[a]

[a]*Laboratory of Molecular Biotechnology, Graduate School of Biological and Agricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan*
[b]*Biomolecular Engineering Research Institute, 6-2-3, Furuedai, Suita, Osaka 565-0874, Japan*

Abstract The heavy chain (Hc) and light chain (Lc) genes of the Fab fragment of a catalytic antibody 6D9 were simultaneously expressed in an *Escherichia coli* in vitro transcription/translation system without a reducing agent. The intermolecular disulfide bond between the Hc and Lc was found formed, suggesting a correct formation of the Fab fragment in the in vitro system. In enzyme-linked immunosorbent assay, the Fab fragment synthesized in vitro exhibited an antigen-binding activity. Addition of reduced glutathione, oxidized glutathione, protein disulfide-isomerase and molecular chaperones, GroEL and GroES, increased the solubility and the antigen-binding activity of the Fab fragment greatly. The in vitro synthesized Fab was purified by means of a hexa-histidine tag attached to the C-terminus of the Hc. Catalytic assay of the purified Fab fragment showed that the His-tagged Fab fragment synthesized in vitro had a catalytic activity comparable to that produced in vivo. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Catalytic antibody; Fab fragment; Cell-free protein synthesis; Antibody activity

1. Introduction

Since a complete antibody such as IgG is a large molecule, much smaller functional domains (Fv, Fab fragments) retaining antigen-binding activity can be applied for a number of practical uses [1,2]. Recent progress in genetic and antibody engineering has made it possible to produce various antibody fragments carrying desirable properties [3]. In addition, as an alternative to the hybridoma technology, cDNA of antibodies can be amplified using appropriate primers, cloned into an expression vector, and synthesized as these small molecules in recombinant *Escherichia coli* to obtain new antibody molecules [4–8].

Because of the small size and useful properties, single-chain Fv (scFv), which is composed of the variable heavy chain (Hc), a linker and the variable light chain (Lc), has been used in a wide range of systems such as phage display and in vivo expression [9,10]. These two variable domains are artificially interconnected by a flexible peptide linker, which is, however, sometimes degraded by proteases, resulting in the dissociation of the two chains. Moreover, the affinity of an original antibody was sometimes impaired in the corresponding scFv [11].

Recently, cell-free protein synthesis systems have been used as an alternative method of producing proteins from cloned genes. This system offers many potential advantages. It can be free from inclusion body formation that sometimes occurs when recombinant proteins are expressed in microbial cells [12]. In addition, two genes can be simultaneously transcribed and translated in an *E. coli* in vitro coupled transcription/translation system, and the two synthesized products can also act on each other [13]. However, only scFv has been reported to be synthesized in the in vitro systems [14,15], and there is no paper describing the Fab fragment formation by a cell-free protein synthesis system until now.

In this report, we describe that a Fab fragment was able to be synthesized in an active form by an *E. coli* in vitro coupled transcription/translation system. The genes encoding the individual chains (Hc and Lc) of the Fab fragment of catalytic antibody 6D9 [11,16] were separately cloned into a T7 expression vector. Both of these plasmids were used as templates for the in vitro system without reducing agent. The synthesized Hc and Lc folded successively to form a Fab fragment with the intermolecular disulfide bond of Hc and Lc showing catalytic as well as binding activity.

2. Materials and methods

2.1. Plasmid construction
The Hc gene was amplified using a set of primers (Hc-1: 5′-GGGAATTC<u>CCATATG</u>CAGGTGCAGCTGCTCGAGTCT-3′ (*Nde*I site is underlined) and ARA-R: 5′-CTTCTCTCATCCGCCAAAA-CAGCC-3′) from the plasmid pARA-6D9Fab [11]. The polymerase chain reaction (PCR) product was digested with *Nde*I–*Kpn*I, and cloned into the *Nde*I–*Kpn*I site of pRSET-B vector (Invitrogen). The resultant plasmid was designated as pRSET-6D9Hc and used for the expression of Hc of 6D9 under the control of the T7 promoter.

The gene of Lc was amplified using Lc-1: 5′-GGGAATTC<u>CA-TATG</u>GAGCTCGTGATGACCCAGACTC-3′ (*Nde*I site is underlined) and ARA-R from the plasmid pARA-6D9Fab. The PCR product was digested with *Nde*I and *Hin*dIII, and cloned into the pRSET-B vector with the *Nde*I–*Hin*dIII site, resulting in pRSET-6D9Lc.

In addition, the Hc gene was amplified using Hc-1 and Hc-2: 5′-CCC<u>AAGCTT</u>ACTAGTACAATCCCTGGGCACAATTTT-3′ (*Hin*dIII site is underlined). The PCR product was digested with *Nde*I–

*Corresponding author. Fax: (81)-52-789 4145.
E-mail address: hnakano@agr.nagoya-u.ac.jp (H. Nakano).

Abbreviations: BSA, bovine serum albumin; CBB, Coomassie brilliant blue; ELISA, enzyme-linked immunosorbent assay; GSH, glutathione reduced; GSSG, glutathione oxidized; Hc, heavy chain of Fab fragment; HPLC, high-performance liquid chromatography; Lc, light chain of Fab fragment; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDI, protein disulfide-isomerase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis
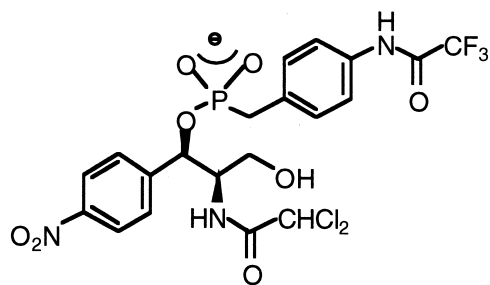
Fig. 1. Structure of hapten used for ELISA assay.

HinDIII and inserted into the NdeI–HinDIII site of pET23b vector (Novagen). The resulting plasmid, pET23b-6D9Hc, contained 6D9 Hc and six histidines at the C-terminus.

E. coli DH5α was used as the host for the DNA manipulator. Nucleic acid sequences of the cloned region of each plasmid were checked with Thermo Sequenase™ II dye terminator cycle sequencing premix kit (Amersham Pharmacia Biotech) and ABIPRISM™ 310 Genetic Analyzer, according to the recommended protocols.

2.2. In vitro coupled transcription/translation

The in vitro coupled transcription/translation system was essentially the same as described previously [17]. Each template DNA of a final concentration of 20 μg/ml was transcribed and translated at 30°C in a 30 μl mixture (56.4 mM Tris–acetate, pH 7.4; 1.2 mM ATP; 1.0 mM each of GTP, CTP, and UTP; 40 mM creatine phosphate; 0.32 mM each, all 20 kinds of unlabeled amino acids; 4% (w/v) polyethylene glycol 6000; 34.6 μg/ml folinic acid; 0.17 mg/ml E. coli tRNAs; 36 mM ammonium acetate; 8 mM Mg(OAc)$_2$; 100 mM KOAc; 10 μg/ml rifampicin; 0.15 mg/ml creatine kinase; 7.7 μg/ml T7 RNA polymerase; and 28.3% (v/v) S30 extract).

The T7 RNA polymerase was purified from E. coli BL21/pREP4/pQE-T7P [18], which was a kind gift from Dr. T. Suzuki. The S30 extracts were prepared from E. coli A19, according to the procedure of Pratt [19] with some modifications as described previously [17]. ^{14}C-Labeled leucine was added to the reaction system at a concentration of 0.016 mM for autoradiography.

Protein disulfide-isomerase (PDI) from Humicola iosolens [20], which was kindly provided by Toyota Central Research and Development Laboratories, was used at a final concentration of 36 μg/ml. Glutathione in the reduced (GSH) and oxidized (GSSG) forms was used at a concentration of 0.1 and 1 mM, respectively. The concentrations of the chaperones in the reaction mixture were as follows: DnaJ, GroEL (TaKaRa, Japan) and GroES (TaKaRa, Japan) 0.4 μM each, and DnaK 1.4 μM. The DnaJ and DnaK were purified from E. coli DH5α/pMAL-C2-DnaJ and E. coli DH5α/pMAL-C2-DnaK [21], respectively, which were kind gifts from Dr. A. Kondo.

2.3. Analysis of solubility of protein synthesized in vitro

After transcription/translation, samples were centrifuged at 15 000 rpm for 5 min to separate soluble (supernatant) and precipitated (pellet) fractions. The pellet was resuspended in the original volume of distilled water. Synthesized proteins existing in the supernatant and in the pellet are defined as soluble and precipitated forms, respectively.
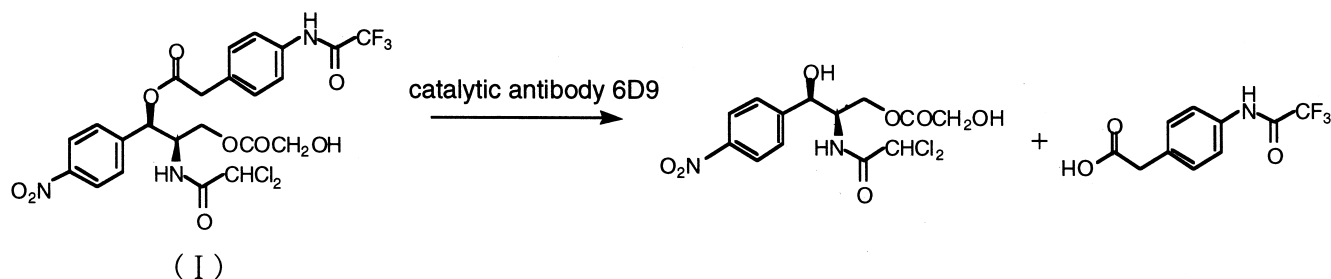
2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography

Typically 5 μl of the samples was boiled with an equal volume of either loading buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, and 0.1% bromophenol blue) for non-reducing conditions, or the loading buffer plus 2% 2-mercaptoethanol for reducing conditions. The boiled samples were electrophoresed on SDS–12.5% polyacrylamide gel, followed by autoradiography.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Falcon Pro-Bind™ assay plates (96-well, Becton Dickinson Labware) were coated with a hapten–bovine serum albumin (BSA) conjugate (50 μl/well, [16]). This hapten was used at 5 μg/ml in 20 mM phosphate-buffered saline (PBS) (3.12 g/l NaH$_2$PO$_4$, 8.8 g/l NaCl, pH 7.4), and its structure is shown in Fig. 1. After incubation at 4°C overnight, the wells were blocked at 37°C for 30 min by 1/4 × Block Ace in distilled water (Dainihon Pharmaceutical, Osaka, Japan), and washed twice with a washing solution (1/10 × Block Ace, 0.05% Tween 20). Cell-free reaction mixtures (30 μl aliquots) were diluted 2000-fold by PBS (pH 7.4). This diluted solution of 50 μl was added into the precoated plate, incubated at 25°C for 2 h, then washed twice with the same washing solution. At the same time, reaction mixtures without DNA template were used for control reactions. Biotinylated anti-mouse IgG (H+L) of 150 μl (5 μg/ml in distilled water; Vector Laboratories, CA, USA) was added to each one and incubated at 25°C for 1 h. After washing twice, ELISA was performed using the Vectastain ABC kit (Vector Laboratories) according to the supplier's protocol. The solution containing o-phenylenediamine and H$_2$O$_2$ was used as the substrate for the peroxidase reaction. The color reaction was stopped by using 2 M H$_2$SO$_4$. Absorbance was measured at 492 nm.

2.6. Purification of Fab synthesized in vitro

In order to purify His-tagged Fab, a HiTrap chelating column of 1 ml (Amersham Pharmacia Biotech) was used. The column was saturated with Ni^{2+} ions by using 0.1 M NiSO$_4$, and then washed with distilled water until Ni^{2+} was eluted. The samples were centrifuged at 10 000 × g for 60 min at 4°C and the supernatant was applied to the column equilibrated with the buffer (50 mM HEPES–KOH, pH 7.6, 1 M NH$_4$Cl, 10 mM MgCl$_2$). The His-tagged Fab was eluted by a linear gradient of imidazole (pH 7.6) from 0 to 500 mM at a flow rate of 2 ml/min. The eluted fraction was concentrated and the buffer was exchanged to 50 mM Tris–HCl (pH 8.0) by using Ultrafree-0.5 Centrifugal Filter Units (Millipore). The protein concentration was determined by measuring the absorbance at 595.5 nm using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, CA, USA) with BSA as standard. The His-tagged Hc synthesized in vitro was also purified by the same method.

2.7. Hydrolytic activity assay

Catalytic activity of 6D9 Fab synthesized in vitro was examined in the hydrolysis of 200 μM substrate shown in Fig. 2 [16], with 2 μM Fab in 50 mM Tris–HCl (pH 8.0) at 25°C. The reaction was initiated by adding 2 μl of a stock solution of the substrate in dimethyl sulfoxide to 18 μl of purified Fab solution. The high-performance liquid chromatography (HPLC) analysis was performed using a CAPCELL PAK C18 UG80 S-5 column (4.6 × 250 mm, Shiseido, Tokyo, Japan) eluted with CH$_3$CN/0.1% aqueous trifluoroacetic acid at a flow rate of 1.0 ml/min with detection at 278 nm. The activities of the 6D9 Fab

Fig. 2. Antibody-catalyzed reaction. The chloramphenicol monoester, substrate (I), was used for the assays of the antibody-catalyzed reaction.
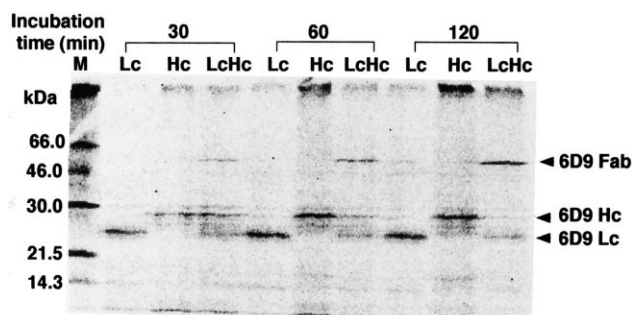
Fig. 3. Autoradiography of the non-reducing SDS–PAGE of Lc, Hc and Fab of 6D9 synthesized in an *E. coli* in vitro coupled transcription/translation system with the plasmids, pRSET-6D9Lc and pRSET-6D9Hc. Samples were taken at 60, 120 and 180 min after the start of incubation.

fragment produced in vivo and purified by Miyashita et al. [11] and the purified His-tagged Hc were also analyzed by HPLC as control.

3. Results and discussion

3.1. Synthesis of 6D9 Fab fragment in the E. coli in vitro coupled transcription/translation system

The cell-free translation system normally contains a reducing reagent such as dithiothreitol to keep the reduced reaction conditions similar to inside a living cell. In order to express the Fab fragment, however, the normal conditions seem inappropriate because disulfide bridges that are necessary to express the functional Fab cannot be formed under such reducing conditions. Therefore, plasmids pRSET-6D9Hc and pRSET-6D9Lc, carrying Hc and Lc genes, respectively, of the Fab fragment of catalytic antibody 6D9, served as templates in the *E. coli* in vitro coupled transcription/translation system without a reducing agent as described in Section 2. The synthesized proteins were analyzed by 12.5% SDS–PAGE under non-reducing conditions and autoradiography. When these two plasmids were used separately, the autoradiography showed that Hc and Lc of 6D9 could be detected as clear bands in the gel with molecular masses of about 26 and 24 kDa, respectively (Fig. 3). When these two plasmids were put into the same reaction tube, in addition to bands from Hc and Lc genes, a new band with an approximate molecular mass of 50 kDa appeared on the gel (Fig. 3). The 50 kDa band was not observed in the early stage of the reaction, but became
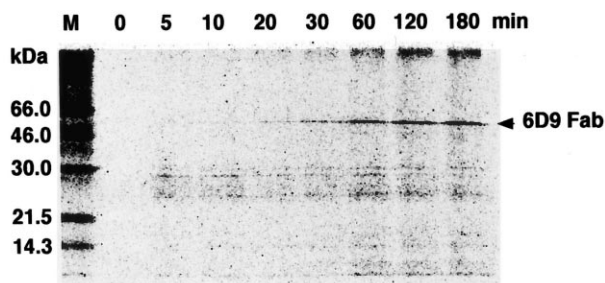

Fig. 4. Time course of 6D9 Fab synthesized in an *E. coli* in vitro coupled transcription/translation system with the plasmids, pRSET-6D9Lc and pRSET-6D9Hc, under non-reducing conditions. The samples were taken at the indicated times after the start of incubation and analyzed by 12.5% SDS–PAGE and autoradiography.
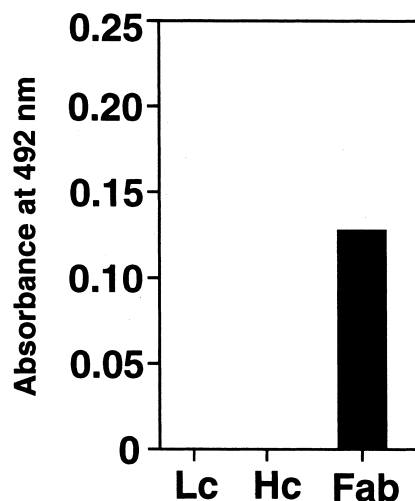

Fig. 5. ELISA analysis of Lc, Hc and Fab of 6D9 synthesized in an *E. coli* in vitro coupled transcription/translation system. Samples were taken at 60 min after the start of incubation.

visible after 20 min of the reaction and intensified afterward (Fig. 4).

These results suggest that the synthesized Hc and Lc have a natural tendency to connect each other with the interchain disulfide bond, resulting in the formation of a Fab fragment in the *E. coli* in vitro coupled transcription/translation system without reducing agent. In addition, the synthesized Fab fragment seemed to be stable, since no degradation was observed during a long incubation period (up to 180 min, Fig. 4).

3.2. Antigen recognition of 6D9 Fab fragment synthesized in vitro

To determine whether this 6D9 Fab fragment synthesized in vitro possesses a correct conformation for antigen-binding, ELISA was carried out against hapten–BSA. As seen in Fig. 5, the ELISA signal of the Fab fragment was significantly as high as 0.13. On the other hand, the signals of the Lc and the Hc were below detection levels. These results strongly suggest that the Fab fragment synthesized in vitro was capable of binding the corresponding antigen.

3.3. Effects of redox potential, PDI and molecular chaperones on the solubility and binding activity of 6D9 Fab fragment

In addition to the interchain disulfide bond, the Fab fragment has also intrachain disulfide bonds within each of the four domains. The conserved intrachain disulfide bond is supposed to be critical for the stability and the activity of antibody domains [22,23], and its formation is a slow process because the cysteine residues are buried in the interior of the protein molecule [24]. Therefore, to minimize the aggregation and misfolding of the Fab, the effects of GSH, GSSG, PDI and molecular chaperones were also examined.

To discover the solubility of the synthesized Fab, the reaction mixture was separated by centrifugation into soluble and insoluble fractions, and analyzed by SDS–PAGE under reducing conditions and autoradiography. Under this condition, only two bands of the expected size for the Hc and the Lc could be seen on the gel (Fig. 6I). To discover the amount of protein in each fraction, the quantity of radiation of each band was measured using a FUJIX BAStation (Fig. 6II).
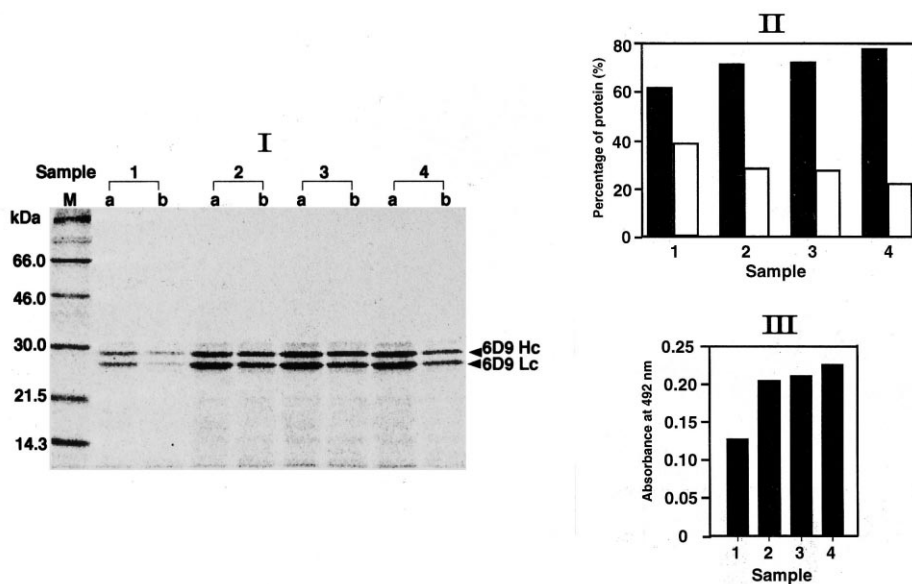
Fig. 6. Effects of GSH, GSSG, PDI and chaperones on the solubility and antigen-binding activity of 6D9 Fab. I: The supernatant (a) and pellet (b) fractions of each in vitro coupled transcription/translation mixture were analyzed by 12.5% SDS–PAGE under reduced conditions and autoradiography. Samples were taken at 60 min after the start of incubation. Sample 1, without addition; sample 2, in the presence of GSH, GSSG and PDI; sample 3, in the presence of GSH, GSSG, PDI, DnaK and DnaJ; sample 4, in the presence of GSH, GSSG, PDI, DnaK, DnaJ, GroES and GroEL. II: Percentage of protein in supernatant (close bar) and pellet (open bar) fractions of each sample of I. III: Antigen-binding activity of each sample of I was analyzed by ELISA as described in Section 2.

The total intensity of the Hc and Lc bands in the reducing gel shown in Fig. 6I was employed as the amount of the Fab fragment present in each sample.

Under our standard reaction conditions, about 60% of the synthesized 6D9 Fab were detected in the soluble fraction. The addition of GSH, GSSG and PDI increased the solubility to 70%. In previous studies, the effects of GSH, GSSG and PDI on the solubility of scFv had been reported [25,26].

It has been shown that molecular chaperone DnaJ and its cofactor DnaK can facilitate the digestion of unfolded proteins by the ATP-dependent protease [27]. Merk and co-workers also reported that the addition of DnaJ and DnaK increased the solubility of scFv [26]. In this work, however, the combinations of DnaJ and DnaK showed no further promotion on the Fab solubility. This difference might suggest that both DnaJ and DnaK families of chaperones bind unfolded proteins in a selective way, which may be dictated by the nature of each protein. On the other hand, the addition of GroEL and its co-chaperone GroES also influenced the folding of the Fab fragment, yielding a further increase in solubility of the Fab fragment by 6%. This result was similar to the previous report about scFv described by Ryabova et al. [14].

From the result of Fig. 6I, the addition of GSH, GSSG, PDI and chaperones also increased the amount of synthesized Fab fragment because the intensity of the bands representing both soluble and insoluble fractions became higher. We also separately examined the effect of GSH/GSSG and PDI. The GSH/GSSG increased both the amount and the solubility of synthesized Fab significantly. The addition of PDI also increased the solubility slightly, but not the amount of the Fab (data not shown). To make a definite conclusion, however, it is necessary to determine further the effects of GSH, GSSG, PDI and chaperones on other proteins.
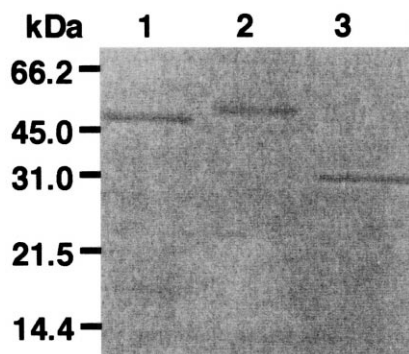

Fig. 7. SDS–PAGE analysis of IMAC-purified 6D9 Fab synthesized in an *E. coli* in vitro coupled transcription/translation system. The gel was stained with CBB. Lane 1, Fab produced in vivo; lane 2, His-tagged Fab synthesized in vitro; lane 3, His-tagged Hc synthesized in vitro.

Table 1
Specific activities of proteins synthesized in vivo or in vitro

Protein (derivation)	Fab quantity (mg)	Activity (units)	Specific activity (units/mg)
Fab (in vivo)	19.4×10^{-3}	35.2×10^{-6}	1.82×10^{-3}
Fab (in vitro)	20.0×10^{-3}	36.6×10^{-6}	1.63×10^{-3}
Hc (in vitro)	10.3×10^{-3}	0.97×10^{-6}	0.09×10^{-3}

We also examined the effects of GSH, GSSG, PDI and chaperones on the antigen-binding activity of the Fab fragment. From the result shown in Fig. 6III, the addition of GSH, GSSG and PDI increased the ELISA signal by 1.6-fold, and that of GroEL and its co-chaperone GroES also increased further to 0.2-fold. On the other hand, there was no influence of chaperones DnaJ and DnaK on the ELISA signal of the Fab fragment. This suggests that the increased solubility of the synthesized Fab fragment is accompanied by a significant increase of antigen-binding activity.

3.4. Catalytic activity of 6D9 Fab fragment synthesized in vitro

To purify the 6D9 Fab fragment, plasmid pET23b-6D9Hc, possessing the 6D9 Hc fused with $6 \times$ His tag sequences at the C-terminal part, was used as the template DNA instead. The pET23b-6D9Hc and pRSET-6D9Lc simultaneously served as template DNA in the same transcription/translation system as above. The synthesized His-tagged Fab fragment was purified with immobilized metal-ion affinity chromatography and checked by SDS–PAGE stained with Coomassie brilliant blue (CBB) (Fig. 7). The His-tagged Fab fragment was detected as a band in the gel with a molecular mass of about 50 kDa. The molecular mass of His-tagged Fab fragment was slightly higher than that of Fab fragment produced in vivo with a molecular mass of approximately 48 kDa. The catalytic activity of the Fab synthesized in vitro was analyzed by HPLC and shown in Table 1. The specific activity of the purified Fab was more than 17 times higher than that of Hc and similar to that of the authentic Fab produced by hybridoma cells. These results indicate that the Fab synthesized in vitro had catalytic activity comparable to Fab fragment produced in vivo.

3.5. Conclusions

In this report we have described a successful synthesis of the Fab fragment of catalytic antibody 6D9 in the *E. coli* in vitro coupled transcription/translation system. This method can provide us with a novel approach for the expression and screening of catalytic antibodies to improve their activities. To our knowledge, this is the first report of the in vitro synthesis of the Fab fragment retaining specific antigen-binding affinity and catalytic activity.

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