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Journal of Chromatography B, 737 (2000) 63–69

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Chromatographic purification of the C_H2 domain of the monoclonal antibody MAK33

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Abstract

The C_H2 domain, one of the constant domains of the murine monoclonal antibody MAK33 (immunoglobulin subtype κ/IgG1) was expressed in *Escherichia coli* forming insoluble inclusion bodies (IBs) and purified by a three-step process including a denaturation–renaturation step, hydrophobic interaction and gel permeation chromatography. After disrupting the cells, the soluble protein fraction was removed by several centrifugation steps. The isolation of the IBs from the cell fragments was achieved by solubilizing the IBs with 6 *M* guanidinium hydrochloride (GdmCl) and 0.1 *M* 1,4-dithioerythrit (DTE) to reduce all disulfide bonds. After refolding the C_H2 domain, 1.5 *M* $(NH_4)_2SO_4$ was added to the protein solution in order to precipitate contaminations. Then the protein was loaded on a Butyl-Sepharose fast flow column and eluted with a linear gradient [1.5–0 *M* $(NH_4)_2SO_4$]. As the last purification step a gel permeation chromatography was run on a Superdex 75 prep grade. Finally, the purity of the C_H2 protein was determined by a silver-stained sodium dodecyl sulfate polyacrylamide gel. We achieved a typical yield of 0.5 mg pure protein per 1 g of wet cells.

Keywords: Purification; Monoclonal antibodies; MAK33

1. Introduction

In contrast to the folding of α-helices, the folding processes which lead to the formation of β-sheet structures are still enigmatic. In this context, an interesting subject of research are all β proteins such as immunoglobulins. The members of the immunoglobulin superfamily exhibit a modular structure. This allows one to dissect the immunoglobulins into heavy and light chains and each of these chains into homologous regions which independently fold to single compact domains. These folding units associate specifically via non-covalent interactions of domain interfaces. Therefore, this protein family represents an ideal model system for analyzing the folding of β-sheet structures.

The domains of immunoglobulins display a folding motif called β-barrel. It consists of two antiparallel β-sheets linked by a buried disulfide bond forming a hydrophobic core. Investigations on the structure formation of several antibody domains have demonstrated that in principle this process is fast. However, during antibody folding the isomerization of proline peptide bonds to the native-state *cis* configuration [1] which is a slow process is the rate-limiting step for the entire folding reaction [2,3].

The most important perequisite for the investigation of protein folding is the ability to obtain sufficient quantities of highly pure and native protein. The expression and subsequent export of re-

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0378-4347/00/$ – see front matter
PII: S0378-4347(99)00377-1

combinant proteins into the periplasm have proved to be a successful way for getting soluble native proteins in large amounts. Up to now, this method was used for the production of several antibodies and their fragments in *Escherichia coli* bacterial strains [4–6]. In contrast to the cytoplasm, the higher redox potential in the periplasm favors the formation of disulfide bridges stabilizing the domain structure of antibodies. Furthermore, antibodies which are toxic for the *E. coli* cells cannot be attacked by proteases [7–9], a process which would diminish the yield.

As an alternative for proteins that do not get expressed well in the periplasm the expression can be achieved in the cytoplasm of *E. coli* by the production of inclusion bodies (IBs) [8]. This method requires a subsequent extraction removing cell debris and the in vitro refolding of the protein aggregates [10]. In general, the protein has to show a reversible unfolding transition. Otherwise native protein cannot be obtained. Moreover, several refolding conditions (temperature, buffer mixture, redox system) have to be tested in order to achieve the best result. If these perequisites are fulfilled and if the expressed IBs are very homogenous, only few purification steps are necessary yielding soluble and pure protein [10]. The Fab fragment of the monoclonal antibody MAK33 was successfully expressed in IBs and refolded [11], whereas the attempt to obtain native Fv fragment (a heterodimer composed of the V_H and the V_L antibody domain) did not succeed [5,12].

In most cases, solubilization and refolding of IBs do not lead to the pure protein of interest straight away because of several contaminants enclosed in the expressed protein aggregates. Chromatography offers a number of techniques which differ in the kind of binding and separation of proteins. Most proteins bind to hydrophobic interaction chromatographic material. Because of the high capacity of hydrophobic interaction chromatography (HIC) adsorbents this method of chromatography is suitable as a capture step for use at an early stage in a purification scheme [13]. Polishing of proteins can be achieved with gel permeation chromatography, a separation technique which was described as early as in 1955 by Lathe and Ruthven [14].

To further investigate β-sheet folding and association in this model system, we cloned the C_H2 fragment (M_r=11 279.7) of the murine monoclonal antibody MAK33 and recombinantly expressed it in the cytoplasm of *E. coli*. MAK33 is directed against the muscle-specific isoform of human creatine kinase (E.C.2.7.3.2.: CK-MM). It was one of the first antibodies to be cloned and expressed in *E. coli* [15]. In vivo, the C_H2 domain contains a carbohydrate moiety that interacts with the carbohydrate of the other C_H2 domain in the antibody. Recombinantly produced C_H2 lacks this feature resulting in monomeric state. Therefore, this domain is an ideal model system to investigate folding without association processes.

In this study, we report on a method for purification of recombinant C_H2 applying inclusion body preparation and a two-step purification by hydrophobic interaction and gel permeation chromatography. Importantly, this is the first time that this domain is available in large amounts allowing intensive thermodynamic and kinetic investigations.

2. Experimental

2.1. Materials

Gene amplification and cloning were performed by using enzymes and buffers from Roche Diagnostics (Mannheim, Germany). Super broth (SB) medium was composed of 20 g/l Bacto Trypton, 10 g/l yeast extract (both from Difco Labs., Detroit, MI, USA), 5 g/l NaCl, 2.5 g/l K_2HPO_4 and 1 g/l $MgSO_4 \cdot 7H_2O$, pH 7.5. Ampicillin, canamycin and acrylamide were obtained from Roth (Karlsruhe, Germany). Lysozyme and DNAse I were from Roche Diagnostics. Triton X-100 was from Merck (Darmstadt, Germany).

2.2. Construction of the C_H2 expression plasmid

The C_H2 gene was obtained by polymerase chain reaction (PCR) amplification. The reaction was performed in 0.5-ml PCR tubes from Biozym (Oldendorf, Germany) in a Primus thermo-cycler from MWG (Ebersberg, Germany). We used the cDNA of the MAK33 γ_1 chain [15] as a template. The primers

prC_H2exp5 and prC_H2exp3 were designed by working with the program Primer Design 1.02 (Scientific and Educational Software, 1990). Thereafter, the C_H2 gene was cloned into a T5 expression vector via *Nde*I and *Xba*I and transformed into the *E. coli* strain HB101 [16,17].

2.3. Expression and isolation of inclusion bodies containing C_H2

The *E. coli* strain HB101 containing the plasmid pAkF-T5/C_H2c+12 was used for the expression of C_H2. The cells were grown in SB medium additionally containing ampicillin and canamycin at 37°C. The expression was started by the addition of 1 m*M* isopropyl β-D-thiogalactopyranoside (IPTG). After 4 h, the cells were harvested by centrifugation (4000 *g*) and an inclusion body preparation was performed according to Ref. [10]. The cell pellet was resuspended in 100 m*M* Tris–HCl, 1 m*M* EDTA, pH 7 at 4°C. Disruption of the cells was carried out by incubation with lysozyme (1.5 mg/g cells) for 30 min at 4°C and subsequent high-pressure treatment. The DNA was removed by the addition of 10 μg/ml DNAse I, 3 m*M* $MgCl_2$ and incubation for 30 min at room temperature. Thereafter, half a volume of 60 m*M* EDTA, 6% Triton X-100, 1.5 *M* NaCl, pH 7 was added and the mixture was incubated for further 30 min at 4°C. Finally, removal of the soluble protein fraction was achieved by centrifugation (40 000 *g*). Washing the IB pellet with 100 m*M* Tris–HCl, 20 m*M* EDTA, pH 7 yielded the C_H2 IBs together with the insoluble cell fragments.

2.4. Inclusion body solubilization [10]

The IB pellet was resuspended in a small volume (1–2 ml/50 mg pellet) of 100 m*M* Tris–HCl, 6 *M* GdmCl, 100 1,4-dithioerythrit (DTE), pH 8. After 2 h of incubation at 25°C, the pH was shifted to a value of 2 by the addition of 1 *M* HCl. The removal of insoluble cell fragments was carried out by a centrifugation step (40 000 *g*). Then, the solution was dialyzed against 4 *M* GdmCl, pH 2 at 4°C and the protein concentration was determined.

2.5. Refolding of C_H2 inclusion bodies

The C_H2 domain was refolded in 0.7 *M* Tris–HCl, 2 m*M* EDTA, 5 m*M* oxidized glutathione (GSSG), pH 8 at 10°C. We applied the pulse renaturation method which is based on the stepwise increase of the protein concentration after each hour. The final C_H2 concentration was 200 μg/ml. Fluorescence (FluoroMax-2 fluorimeter with a 1 cm stirred and thermostated cell; Spex, Edison, USA) and circular dichroism (J 715 spectropolarimeter with a PTC 343 Peltier unit; Jasco, Tokyo, Japan) measurements were performed to check whether refolding was successful and the antibody domain had regained its native conformation.

2.6. Chromatographic purification

As a first column we chose a Butyl-Sepharose fast flow column (volume 15 ml, 7.5 cm×1.6 cm) from Amersham Pharmacia (Uppsala, Sweden). Therefore, ammonium sulfate was added to the solution containing refolded C_H2 until a final concentration of 1.5 *M* was reached. The column was equilibrated with 100 m*M* Tris–HCl, 2 m*M* EDTA, 1.5 *M* ammonium sulfate, pH 7. Elution of bound C_H2 was performed by a linear gradient from 1.5 to 0 *M* ammonium sulfate.

Pure C_H2 was obtained after a gel permeation chromatography run on a Superdex 75 prep grade column (volume 120 ml, 60 cm×1.6 cm) from Amersham Pharmacia. The buffer was 100 m*M* Tris–HCl, 2 m*M* EDTA, 300 m*M* NaCl, pH 7. All the column runs were carried out on a HighLoad system from Amersham Pharmacia at 4°C.

2.7. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

To test the purity of the C_H2 domain SDS–PAGE was performed after each purification step. We used a LKB 2050 Midget gel electrophoresis unit connected to a EPS 600 power supply, both from Amersham Pharmacia. The size of the gels was 7×6 cm with a thickness of 0.75 mm. The acrylamide quota in the collecting gel was 5% and in the separation gel 15%. Gels were run at a constant current of 25 mA and at

room temperature. The running buffer contained 25 m*M* Tris–HCl, 200 m*M* glycine and 0.1% SDS (pH 8.8). After the run the silver-staining procedure was performed according to Ref. [18].

3. Results and discussion

3.1. Expression of C_H2 in Escherichia coli

For the preparative production of the antibody domain C_H2, the *E. coli* strain HB101 [16,17] containing a plasmid with the C_H2 gene under the control of a T5 promoter was grown in 2 l SB medium with additional amounts of ampicillin and canamycin. This medium proved to be better than normal Luria Broth (LB) medium [19] since higher cell densities and therefore higher yields of C_H2 could be achieved. The domain was cloned with 12 additional polar or charged amino acids at the C-terminus to increase the solubility of the protein. Expression was achieved by the addition of 1 m*M* IPTG at 37°C. Since the temperature causes a high expression rate and the cytosol of *E. coli* represents a reducing environment, disulfide bonds cannot be formed and the protein is produced as inclusion bodies. Hence, proteases which occur in the cytosol are not able to attack the C_H2 domain. Moreover, the protein is not toxic for the cells because of the fast aggregation [7–9]. After 4 h the expression rate could not be increased any more (Fig. 1A). The amount of C_H2 was 25% with respect to the entire protein amount of the *E. coli* cell. This value was even obtained for other proteins produced in IBs [20]. Analyzing the soluble and the insoluble protein fraction showed that the C_H2 domain was present as insoluble IBs (Fig. 1B).

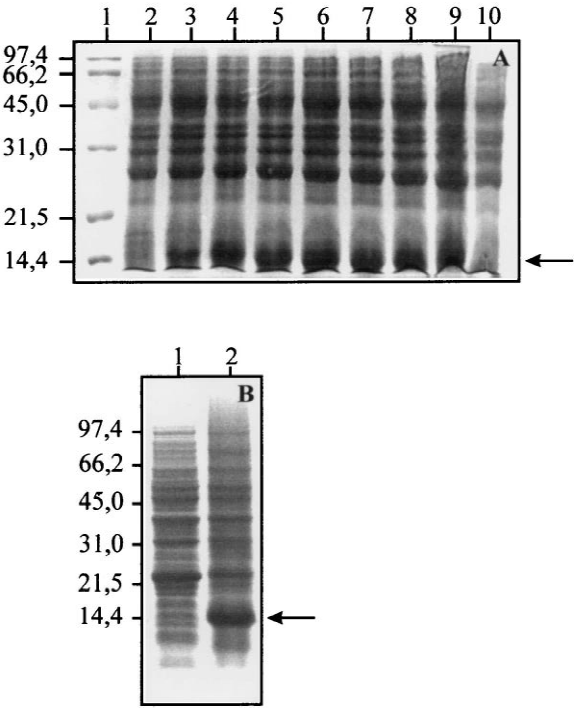

Fig. 1. Expression of recombinant C_H2. (A) Expression kinetic of C_H2 in *E. coli* HB101. (1) Low-relative-molecular-mass standard; (2) cell culture without the expression plasmid; (3) 1 h after induction; (4) 2 h; (5) 3 h; (6) 4 h; (7) 5 h; (8): 6 h; (9) 18 h; (10) cell culture without the expression plasmid. (B) Protein distribution after cell disruption: soluble (1) and insoluble (2) proteins (15% polyacrylamide gel, silver-stained). Numbers indicate relative molecular mass $\cdot 10^{-3}$.

3.2. Isolation and solubilization of inclusion bodies

Since IBs have a relatively high density, it is possible to separate them from the soluble protein fraction and the cell fragments by centrifugation. Maximum cell disruption can avoid the co-sedimentation of IBs and cell fragments. Therefore, cells were opened by treatment with lysozyme, a subsequent high pressure disruption and finally by the addition of tensides such as Triton X-100. This procedure proved to be very effective. Highly homogenic C_H2 IBs could be obtained.

In spite of the high amount of secondary structure, IBs are not soluble under physiological conditions. Therefore, solubilization was performed in the presence of a strong denaturing reagent as it is represented by GdmCl [10]. Urea solutions are not useful for denaturation in this context because they contain small amount of isocyanide which can react with lysine amino acid side chains. Since C_H2 possesses one disulfide bridge and thus the IB aggregates are intermolecularly crosslinked via the cysteine residues, 100 m*M* DTE was added to break these linkages. This reducing reagent was removed by

dialysis afterwards. The yield of solubilized C_H2 was 183 mg/l liquid culture [$O.D._{600(HB101)}$=2.3].

3.3. Refolding of C_H2

The renaturing of unfolded proteins can be achieved in two different ways. First, the dilution into native buffer is practicable if the protein does not tend to aggregate under physiological conditions. Removal of the denaturant by dialysis represents the other possibility. However, here, the protein is under denaturing conditions for a long time. Therefore, protein might be lost due to unstable folding intermediates [10].

Refolding of the C_H2 domain was initiated by the dilution into native buffer. Since the antibody domain contains one intramolecular disulfide bond, no native protein was obtained without any redox system in the refolding buffer (data not shown). The addition of 5 m*M* GSSG markedly increased the yield of native C_H2 (Fig. 2A). However, additional amounts of reduced glutathione (GSH) in the solution did not have any effect (data not shown). Increasing the concentration of C_H2 in the refolding buffer by the Puls renaturation method resulted in no significant lost of protein by aggregation (Fig. 2B). The best yield of native C_H2 (25%) was finally achieved by performing refolding at 200 μg/ml.

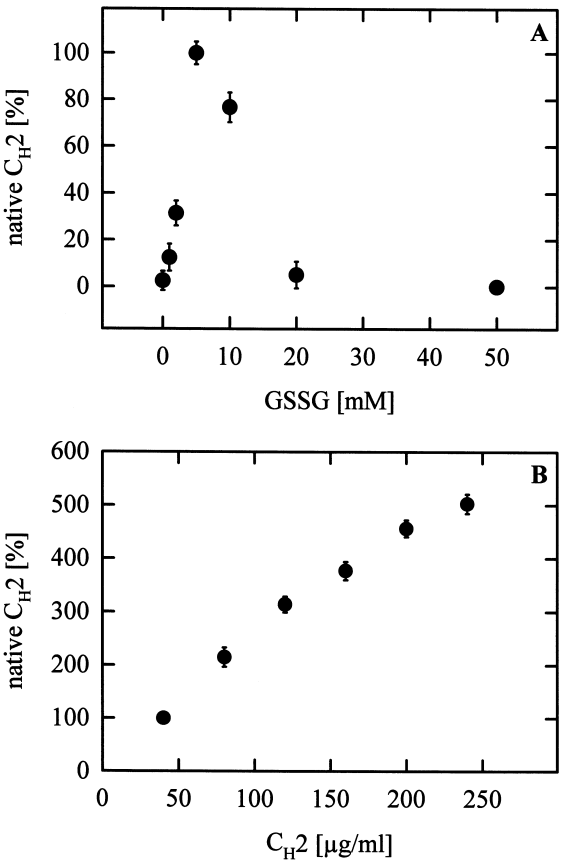

Fig. 2. Refolding yield of C_H2 in dependence on different parameters. (A) Variation of the GSSG concentration. (B) Renaturation at different protein concentrations.

3.4. Purification of recombinant C_H2

Analyzing the amino acid composition of C_H2 via looking at the DNA sequence demonstrated that this antibody domain contains a large number of hydrophobic residues. These amino acids form a large hydrophobic patch on the surface where the sugar moiety is supposed to be in natural C_H2 [1]. Therefore, we chose a Butyl-Sepharose fast flow column for the first purification step. This column proved to be more effective than Phenyl-Sepharose (data not shown). During loading the column the C_H2 domain completely bound to the column. C_H2 was eluted by a linear salt gradient between 0.3 and 0 *M* ammonium sulfate (Fig. 3A). Only one band of higher relative molecular mass could be detected on a SDS–PAGE (Fig. 3C). Additionally, we could use this purification step for concentrating the refolding solution. For separating the C_H2 domain from this one major contaminant, we performed gel permeation chromatography runs on a Superdex 75 prep grade column because of the great difference in the relative molecular mass. Furthermore, changing the buffer conditions for purification steps by anion- or cation-exchange chromatography (data not shown) could be avoided by using this column. The identity of the C_H2 domain was verified by Edman degradation [21].

Taken together, C_H2 could be purified from inclusion bodies expressed in *E. coli*. Refolding and simultaneous formation of the intrachain disulfide bond was achieved with a yield of 25%. HIC, namely Butyl-Sepharose, proved to be the most efficient step in this purification. This purification

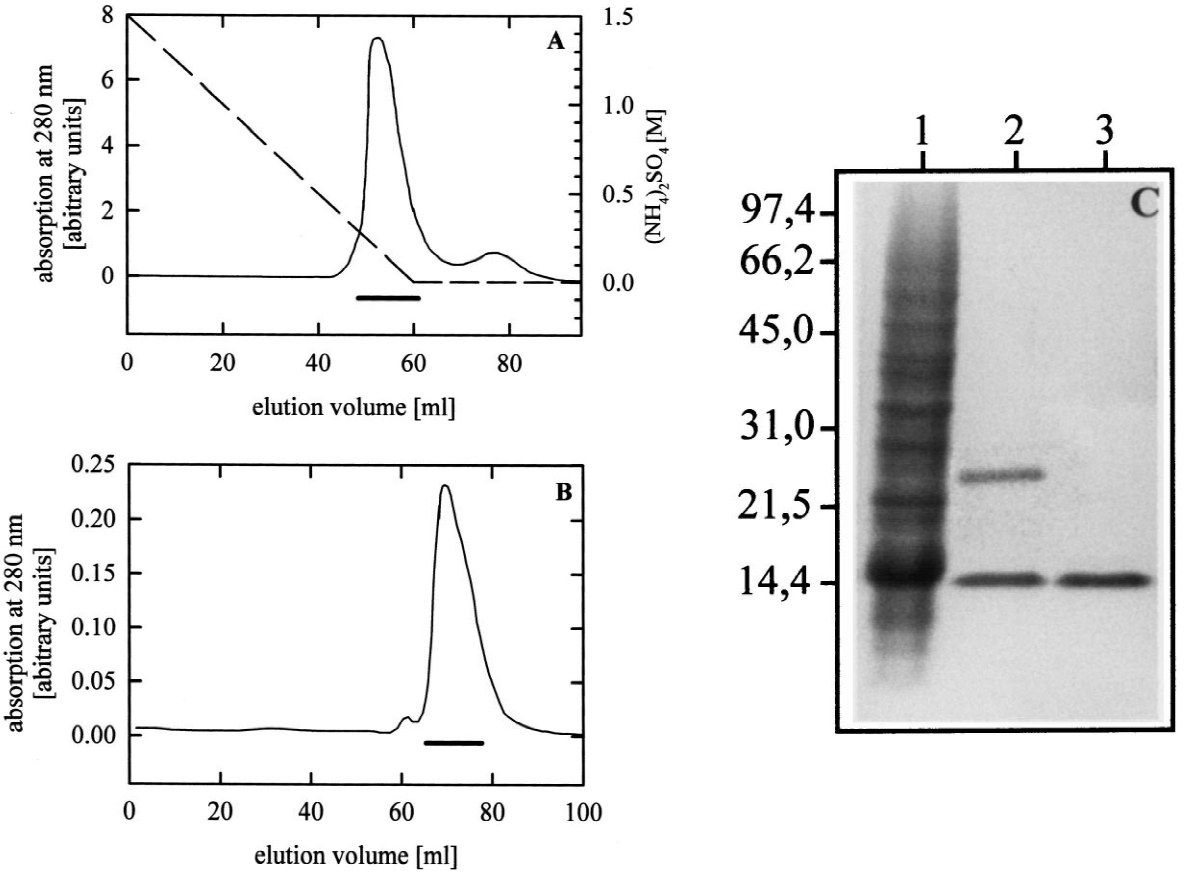

Fig. 3. Purification of C_H2. (A) Elution profile of the Butyl-Sepharose fast flow column. The buffer conditions were 100 m*M* Tris–HCl, 2 m*M* EDTA, pH 7. The column run was carried out at a flow-rate of 2 ml/min at 4°C. (B) Elution profile of the gel permeation chromatography run on a Superdex 75 prep grade column. The flow-rate of the buffer containing 100 m*M* Tris–HCl, 2 m*M* EDTA and 300 m*M* NaCl at pH 7 was 0.3 ml/min. The elution of protein was monitored by measuring the absorbance at 280 nm. (C) C_H2 purification gel (15%, silver-stained). (1) Protein content after cell disruption; (2) pool from the Butyl-Sepharose; (3) pure C_H2 after the gel permeation chromatography run. Numbers indicate relative molecular mass $\cdot 10^{-3}$.

procedure yielded enough protein of sufficiently high purity to carry out the planned investigations in protein folding concerning the behavior of β-sheet structures and disulfide bond formation.

Acknowledgements

We thank Johannes Buchner for supporting these studies. Furthermore, we are grateful to Carolin Kleylein and Christian Mayr for stimulating discussions and critically reading the manuscript.

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