Biotechnol. Bioprocess Eng. **1998**, 3, 82-86

Single-Step Purification of Proteins of Interest from Proteolytically Cleaved Recombinant Maltose-binding Protein (MBP) Fusion Proteins by Selective Immunoprecipitation of MBP

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The maltose binding protein (MBP) fusion protein system is a versatile tool to express and isolate recombinant proteins in *E. coli*. In this system, MBP fusion proteins are efficiently isolated from whole cell lysate using amylose conjugated agarose beads and then eluted by competition with free maltose. Since MBP is a rather large molecule (~42 kDa), for further experiments, the MBP part is usually proteolytically cleaved from the fusion protein and subsequently removed by ion-exchange chromatography or rebinding to amylose columns after washing out excess and MBP-bound maltose. In the present study, we have developed an improved method for the removal of cleaved MBP, which is advantageous over conventional methods. In this method, factor Xa cleaved MBP fusion proteins were incubated with Sepharose beads conjugated with MBP specific monoclonal antibodies and then precipitated by centrifugation, resulting in highly purified proteins in the supernatant.

Key words: maltose binding protein (MBP); factor Xa; recombinant protein; protein purification; anti-MBP monoclonal antibody

INTRODUCTION

There has been described a variety of expression systems for the recombinant production of genetically engineered proteins in *E. coli*. Among them, the maltose binding protein (MBP) fusion protein system [1] is unique in its flexibility as well as in conferring solubility to the fused protein of interest. Actually, the MBP-, together with the thioredoxin-fusion protein system [2, 3] is one of the most effective systems reported so far for the soluble expression of recombinant proteins which are otherwise expressed only as inclusion bodies [4]. Furthermore, the MBP-fusion protein system is also attractive in its simplicity and effectiveness regarding the purification of recombinantly expressed fusion-proteins, since it provides the possibility of a single-step isolation of MBP-fusion proteins using a simple column-binding and -elution protocol.

In its essence, the MBP-fusion protein expression system consists of an antibiotically selectable plasmid vector into which the *malE* gene of *E. coli* is inserted under the control of an inducible promoter [5]. In frame to the *malE* gene, the protein of interest is fused and expressed in *E. coli*. For the isolation of MBP fusion proteins, amylose (a multimeric form of maltose)-conjugated agarose resin is used, which is packed into a column and used to select MBP containing proteins from the bacterial cell lysate [6]. Specifically bound proteins are then eluted using free maltose, which competes with the resin-conjugated amylose for MBP. In the commercially available versions of this system, several improvements have been introduced [7]. They include among others, the targeted expression of MBP-fusion proteins in the cytoplasm by removal of the signal peptide of MBP (i.e. pMAL-c2 vector, New England Biolabs, Beverly, MA) or the insertion of multiple cloning sites for the convenient subcloning of cDNA of proteins of interest, and so on. But the most prominent feature of these vectors is the integration of a protease sensitive site right after the MBP sequence which enables the proteolytic splicing of the fused protein from the original MBP-fusion protein. Since the relatively large mass of MBP (~42 kDa) often represents a hindrance in analyzing the functional aspect of the fused protein [8, 9], the cleavage and removal of MBP has been regarded as a prerequisite for a full evaluation of the functional and structural aspect of the protein of interest.

While the cleavage of MBP-fusion proteins is quite simple, on the other hand, the subsequent removal of the digestion products and contaminations, i.e. MBP and uncleaved intact fusion proteins, often represent a complication in the purification of the protein of interest. Two methods have been generally used so far for the separation of MBP and the pro-

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cessed fusion protein. One is the binding onto and elution from ion-exchange chromatography columns, and the other the rebinding of cleaved MBP-fusion proteins to amylose resins by which the protein of interest is found as free form in the flow-through [10]. Neither of these methods is satisfactory in the convenience of the protocol nor in the simplicity of the procedure. Furthermore, the multiple steps engaged in the removal of the spliced MBP proteins often result in the loss of purified proteins thereby complicating protein isolation especially in small scale (< 100 μg). Because of such reasons, a new method for the removal of cleaved MBP products has been awaited that could overcome the difficulties as mentioned above while maintaining the purity and reproducibility of the conventional methods.

The generation of a novel MBP-specific murine monoclonal antibody termed HAM-19 [8] opened a new way for the handling of the proteolytically spliced MBP proteins. In regarding MBP not as a functional moiety but as a simple tag, the cleaved MBP products and even still uncleaved MBP-fusion proteins could be removed by immunoprecipitation. Using batch procedures or immunoaffinity columns, the unbound free fractions which will contain the MBP-free proteins of interest might be isolated as supernatant or flow-through, respectively.

In the present study, this concept was worked out using a fusion protein of MBP and a partial region of the hepatitis B-virus preS1 envelope protein, the preS1(1-56). This specific protein of interest has been described to play an important role in the host cell binding of the hepatitis B virus [11] as well as has been reported to contain major immunological determinants [12]. The successful isolation and purification of the MBP-free form of the preS1 (1-56) polypeptide will be of great interest in further studies regarding the biological role of this specific sequence. Since the MBP-preS1(1-56) fusion protein was originally cloned and expressed in the pMAL-c2 vector, the preS1(1-56) region can be separated by factor Xa digestion and be isolated by subsequent purification steps. A practical protocol and possible further applications are suggested.

MATERIALS AND METHODS

Expression and Purification of MBP-Fusion Proteins

The expression vector coding for a fusion product between MBP and the hepatitis B virus preS1(1-56), subtype *adr*, had been previously described [13], and was a kind gift of Dr. H. J. Hong, Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea. This vector was transformed into *E. coli* (strain DH5 α) and induced for expression in their log growth phase with 0.3 mM IPTG for 3 hours. The MBP-fusion protein was isolated from the soluble fraction of the sonicated cell lysate either by passage over an amylose column (New England Biolabs) and the subsequent elution with 20 mM of free maltose or alternatively over a DEAE-Sepharose CL-6B column, as previously described [14]. The identity and purity of the eluted fusion protein was confirmed by SDS-polyacrylamide gel electrophoresis (PAGE).

Factor Xa Cleavage of MBP-Fusion Protein

For cleavage of the HBV-preS1(1-56) fragment from the MBP-preS1(1-56) fusion protein, the MBP-fusion proteins were incubated with 1/100 (w/w) amount of factor Xa (New England Biolabs) for 48 hours at 16℃ in the same buffer as used for elution. Complete cleavage of the fusion protein was confirmed in a tricine-SDS PAGE [15].

Generation of Anti-MBP Immunoaffinity Resins

HAM-19 ascites fluid was obtained from pristane treated BALB/c mice, which were injected with hybridoma cells producing the anti-MBP specific mAb HAM-19 [8]. After centrifugation for 30 min at 10,000 g, the ascites supernatant was filtrated through a Whatman paper (Whatman Intl. Ltd., Maidstone, England) and an 0.2 micron cellulose acetate filter (Corning Glass Works, Corning, NY), and finally diluted with the same volume of PBS (pH 8.0). The immunoglobulin fraction was isolated using protein A Sepharose. For the conjugation to CNBr-Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden), firstly, purified antibodies were buffer changed into coupling buffer (0.5 M NaCl, 0.1 M $NaHCO_3$, pH 8.3), and the CNBr Sepharose beads were activated with 1 mM HCl. After washing the activated beads in coupling buffer, the beads were mixed with the antibody solution and incubated overnight at 4℃ under continuos rotation. The next day, Sepharose beads were washed extensively with PBS (pH 8.0) and the beads collected. The ratio of the amount of bound antibodies to 1 ml of CNBr activated Sepharose bed volume was determined to be of 2 mg/ml.

Removal of MBP and Intact Fusion Proteins From the Factor Xa Cleaved MBP-Fusion Protein Mixture

The factor Xa digested MBP fusion protein solution was mixed with HAM-19 conjugated CNBr-Sepharose to a ratio of 100 μg MBP to 50 μl bed volume of HAM-19 Sepharose. The binding reaction was performed in a PERFECTprep™ Spin Column device (5 Prime -> 3 Prime Inc, Boulder, CO), which consists of membrane filter bottomed empty column inserted into an 1.9 ml collection tube. The mixture was then incubated for 3 hours under continuous rotation, and thenafter, the beads were down centrifuged for 3 min at 250 g. The flow-through was transferred into a new Eppendorf tube, and the beads were washed once again with the same volume of ddH_2O. The successful recovery of the proteolytically cleaved protein of interest was confirmed by tricine-SDS-PAGE.

RESULTS AND DISCUSSION

The MBP-preS1(1-56) fusion protein contains a factor Xa sensitive site between each protein domains [16]. The MBP part is located to the N-terminal of the fusion protein, while the amino acid 1-56 region of the hepatitis B virus preS1 envelope protein lies downstream to the factor Xa recognition site. Right after this sequence is the multiple cloning

site, into which cDNAs for the expression of proteins of interest can be inserted in frame to MBP. For the MBP-preS1(1-56) fusion protein as well as for every other MBP-fusion proteins, there is often the need to remove the MBP portion for performing further studies with the originally fused protein of interest. This is the case when using the protein of interest for immunization as well as when a free N-terminus is required for the functional characterization of the fused protein. While it is true that the MBP region confers high solubility to the fusion proteins [4], it is also obvious that the bulky MBP domain could hinder the proper folding of the fused protein, which might be essential in exerting its natural function. The generation of improved linker sequences between MBP and the fusion protein has greatly widened the choice for methods in the cleavage of the MBP, enabling the usage of various proteases like enterokinase and genenase™ I in addition to the originally described factor Xa [7]. Nevertheless, there is still the problem of accomplishing an effective removal of the cleaved MBP. Recommended, and also the only protocols that have been described so far, are the purification methods in which either the reaction with the desired proteins is dialyzed into low salt buffer, then bound and eluted using ion-exchange chromatography in a salt gradient, or where free- and MBP-bound maltose are removed over a hydroxypatite column and then the proteins are rebound and eluted with amylose resins, once again. However, neither of these methods is satisfactory in their efficiency nor in their simplicity. Both methods require buffer changes of the eluted proteins as well as column packing and fraction collections of the purified proteins. Furthermore, these multiple step and column passage procedures involve some unavoidable loss of the proteins together with an increase of the elution volume, which make these methods inappropriate for the removal of cleaved MBP especially in the mini-scale preparation of proteins.

To overcome the inconveniences and inefficiencies of the systems as described above, a novel approach was developed for the MBP free preparation of proteins in interest. In the present study, the efficient elimination of proteolytically spliced MBP and some even still intact MBP-fusion proteins were achieved by a new protocol which involves the immunoprecipitation of MBP and MBP-fusion proteins using anti-MBP antibody conjugated Sepharose beads. To show the effectiveness of this system, first, MBP-preS1(1-56) fusion proteins were successful expressed and purified from *E. coli* transformed with the corresponding expression vector (Fig. 1) as described in Materials and Methods. A single band of about 50 kDa was identified in the purified fraction, which is identical to the theoretical molecular weight of this MBP-fusion protein. The purified proteins were then digested with factor Xa in the same buffer for 48 hours at 16℃, and the successful was confirmed in a tricine SDS-PAGE (Fig. 2). For the removal of cleaved and still intact MBP-fusion proteins, anti-MBP antibody conjugated Sepharose beads were prepared. The monoclonal antibody (HAM-19) used in this study is a mouse IgG1 antibody which generation and characterization have been previously described [8], and that is conditionally available

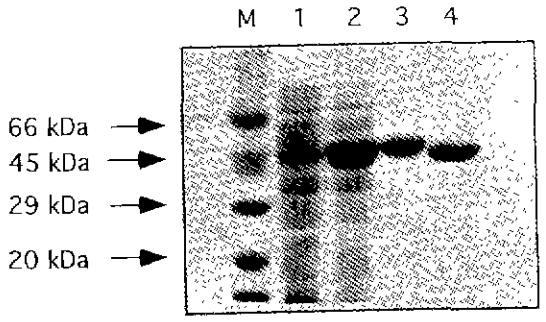

Fig. 1. Expression and purification of MBP-preS1(1-56) protein. Lane M, molecular weight marker; lane 1, uninduced cells; lane 2, IPTG-induced cells; lane 3, purified MBP-preS1(1-56) proteins, lane 4, pure MBP.

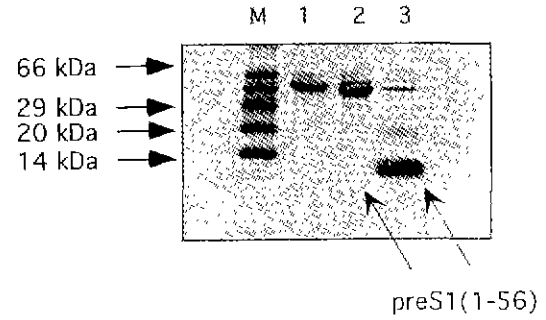

Fig. 2. Purification of factor Xa cleaved preS1(1-56) by the newly described single-step procedure. Lane M, molecular weight marker; lane 1, purified intact MBP-preS1 (1-56); lane 2, factor Xa cleaved fusion proteins; lane 3, purified MBP-preS1(1-56).

from the Korean Type Culture Collection (KCTC), Yusong, Taejon. As described in Materials and Methods, HAM-19 was isolated from mouse ascites fluid and bound to Sepharose beads.

The isolation of protease cleaved proteins of interest, in this case the HBV-preS1(1-56) fragment, was achieved by immunoprecipitation of MBP or MBP-fusion proteins with the prepared HAM-19 beads. For this, the protease reaction was directly mixed with antibody coated beads to a ratio of 10 μl beads for 5 μg MBP-fusion proteins and incubated for 1 hour under continuous rotation at RT. Thenafter, beads were shortly down centrifuged in a filter membrane supported spin column device, and the flow-through containing purified HBV-preS1 fragments was collected. In Fig. 2 is shown that this method indeed ensures the effective elimination of cleaved MBP as well as still intact MBP fusion proteins from the protease treated reaction. It is evident into which extent this protocol is superior to the conventional methods as have been described so far [10]. Firstly, as shown in Fig. 3, the whole procedure is remarkably shorter than the method using rebinding to amylose resins (Fig. 3 left panel) and even more simple than that by ion-exchange chromatography (procedure not shown), since this novel method involves actually only a single step for the removal of contaminating MBP products. Also, the isolation of the proteins of interest can be performed in the same reaction as used in the protease treatment, since there is no need for buffer change into low salt buffer (for ion-exchange chro-

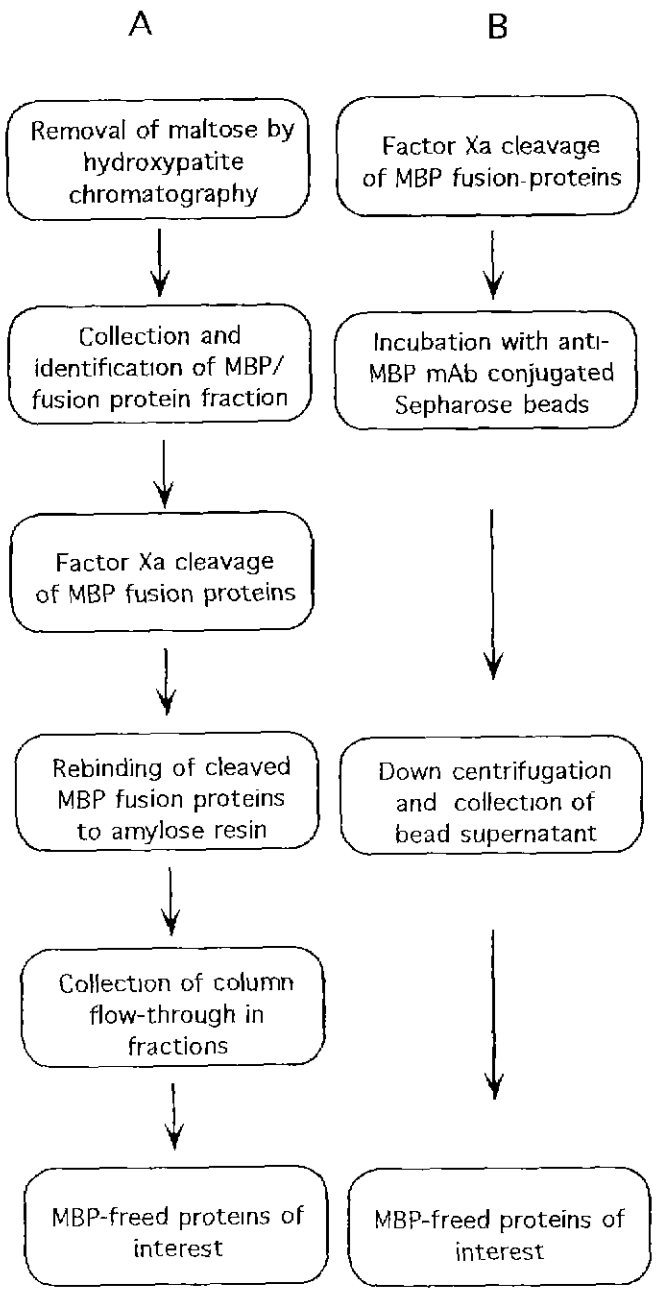

Fig. 3. Comparison of the conventional method to the protocol as described in this study for removal of proteolytically cleaved MBP and intact MBP fusion proteins. A: a conventional method for the purification of proteolytically cleaved proteins of interest from MBP-fusion proteins, B: the new purification method as described in this study.

matography) or removal of free- and MBP-bound maltose (for re-binding to amylose resins). To the second, also the smallest amount of MBP-fusion proteins can be used as starting material for purification, since this novel approach does not require the packing of and elution from columns which is, however, the case for the other conventional methods and result in the unavoidable non-specific loss of the starting material. In summary, in the present study, an improved method has been described by which undesired protease cleavage products are effectively removed from a mixture of MBP and the cleaved proteins of interest, which were previously recombinantly expressed in form of MBP-fusion proteins. Beside to provide a more efficient and convenient way to isolate cleaved proteins of interest, this method even more enables the handling of minute quantities of cleaved proteins, which are more readily required in the analytical assays of protein functions.

Acknowledgements This work was supported in part by a grant (HS2090) of the Ministry of Science and Technology, Korea.

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