Journal of Biochemistry and Molecular Biology, Vol. 31, No. 5, September 1998, pp. 427–431

JOURNAL OF
Biochemistry and Molecular Biology

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Expression of an Angiogenin Binding Peptide and Its Anti-Angiogenic Activity

Suk-Jung Choi*, Miwon Ahn, Kyoung-Bum Yoon and Jong-Won Park
Department of Chemistry, Kangnung National University, Kangnung 210-702, Korea

Received 2 March 1998, Accepted 14 May 1998

In the previous report (Choi *et al.*, 1997), the angiogenin binding peptides identified from a phage-peptide library were analyzed by using the fusion proteins composed of the *Escherichia coli* maltose binding protein and its corresponding peptides. However, it was difficult to obtain a sufficient amount of the fusion proteins required for further analysis because of the low expression level. We now report a high level expression of the fusion protein and analysis of its anti-angiogenin activity. The use of strong T7 promoter and removal of signal sequence allowed about a 20-fold increase in the expression efficiency of the fusion protein. We were able to obtain about 10 mg of purified fusion protein from one liter of culture. The purified fusion protein showed angiogenin-specific affinity and inhibited the binding of biotinylated actin to human angiogenin at IC_{50} of 0.6 mM. Its anti-angiogenin activity was also revealed by the chorio-allantoic membrane assay.

Keywords: Angiogenin inhibitor, Maltose binding protein, Phage-peptide library.

Introduction

Angiogenesis is the growth of new capillaries from preexisting blood vessels, and is a fundamental process in the formation of the vascular system during embryonic development. In adulthood, tissue regeneration and reorganization, as well as several pathological conditions including growth and metastasis of solid tumors, have been shown to be dependent on neovascularization (Fidler and Ellis, 1994; Hanahan and Folkman, 1996). The angiogenic process includes the local degradation of the basement membrane surrounding the endothelial cell tube, invasion of the endothelial cells into the surrounding stroma, and proliferation of the endothelial cells. It is mediated by several angiogenic factors released by both tumor cells and host cells.

The inhibition of angiogenesis is an attractive therapeutic target for the treatment of both primary and metastatic cancers, and various anti-angiogenic agents have been identified (Baillie *et al.*, 1995). Most of the agents are known to act on the endothelial cell or endothelium-extracellular matrix interaction. However, it would be more plausible to block the first step of the process through inhibiting the action of angiogenic factors.

Angiogenin is one of these angiogenic factors, and was first isolated from a human adenocarcinoma cell culture medium (Fett *et al.*, 1985). Its amino acid sequence (Kurachi *et al.*, 1985; Strydom *et al.*, 1985) and crystal structure (Acharya *et al.*, 1994; 1995) show a structural similarity to pancreatic ribonuclease A (RNase A). It was expressed in *Escherichia coli* (Shapiro *et al.*, 1988; Jung and Choi, 1997) as well as in a eukaryotic cell (Kurachi *et al.*, 1988). Both of its ribonucleolytic and angiogenic activities are abolished by the human placental ribonuclease inhibitor (Shapiro *et al.*, 1986; Shapiro and Vallee, 1987). Angiogenin has the ability to bind to the cell-surface actin of endothelial cells (Hu *et al.*, 1991; 1993). Its angiogenic activity can be inhibited by substances blocking the binding of angiogenin to the cell-surface actin, such as soluble actin (Olson *et al.*, 1995; Chang *et al.*, 1996), peptides complementary to the actin-binding site of angiogenin, (Gho *et al.*, 1997), or a peptide identified from a phage-displayed peptide library (Gho and Chae, 1997).

In our previous work, we identified a high affinity angiogenin-binding peptide from a phage-displayed peptide library (Choi *et al.*, 1997). Phages bearing these angiogenin-binding peptides were selected by estimating the affinity of each phage after a biopanning process. The

* To whom correspondence should be addressed.
Tel: 82-391-640-2306; Fax: 82-391-647-1183
E-mail: sjchoi@knusun.kangnung.ac.kr

peptides were expressed in *E. coli* as fusion proteins with the *E. coli* maltose binding protein (MBP), and the affinity of the peptides were estimated quantitatively with the fusion proteins. One of the fusion proteins had a high affinity to angiogenin with a dissociation constant of 60 nM and it showed no apparent sequence or hydropathic profile homology with the peptides obtained by Gho (Gho and Chae, 1997; Gho *et al.*, 1997). In our construct, however, the yield of the fusion proteins was not satisfactory for the large-scale preparation. Here, a high yield expression of the peptide–MBP fusion protein is described. The purified fusion protein inhibited the angiogenic activity of angiogenin and its interaction with actin.

Materials and Methods

Construction of an expression vector for the peptide–MBP fusion protein, AMBP15 A new expression vector, pRAMBP15, was constructed for the more efficient expression of the AMBP15 fusion protein as illustrated in Fig. 1. The previously constructed vector, pAMBP15 (Choi *et al.*, 1997) was digested with *Nae*I and *Eco*RI, and a fragment containing the angiogenin-binding peptide and the *mal*E gene (1.2 kb) was purified. The fragment was ligated into the pRSETA vector (Invitrogen, San Diego, USA) which was digested with *Nhe*I and *Eco*RI, and dephosphorylated with calf intestinal alkaline phosphatase (CIP). The protruding end generated by *Nhe*I digestion was converted into a blunt end with the *E. coli* DNA polymerase I large fragment and four deoxyribonucleotides before the CIP treatment. Correct clones were selected by the restriction enzyme digestion analysis. The signal sequence of gene III was completely removed by this cloning scheme because the *Nae*I site is located at the beginning of the gene encoding the mature peptide–gene III fusion protein.

Expression and purification of AMBP15 *E. coli* BL21(DE3) pLysS was transformed with the constructed pRAMBP15 plasmid, and was grown in 200 ml of LB medium containing 0.2% glucose and 100 μg/ml ampicillin. The AMBP15 protein synthesis was initiated by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM. The culture was further incubated at 37°C for 4 h with shaking. The cells were harvested and resuspended in 20 ml of C buffer (20 mM Tris·Cl, pH 7.4, 0.2 M NaCl, 1 mM EDTA). The suspension was sonicated after freezing at −70°C and thawing at 37°C. The sonicated material was centrifuged at 18,000 × *g* for 30 min to remove cell debris, and the clear supernatant was applied to a 1.5 cm × 6 cm amylose column (NEB, Beverly, USA) equlibrated with C buffer. The column was washed with 20 ml of C buffer, and the bound protein was eluted with E buffer (10 mM maltose in C buffer). The protein was concentrated with Centriprep-10 (Amicon, Beverly, USA) and analyzed by 10% sodium dodecylsulfate-polyacrylamide gel electropohoresis (SDS-PAGE). The expected molecular mass of the resulting fusion protein was about 45 kDa.

Enzyme immunoassay analysis of AMBP15 The binding of AMBP15 to angiogenin or RNase A was analyzed by the enzyme immunoassay method as previously described (Choi *et al.*, 1997). The inhibition activity of AMBP15 on the binding of actin to angiogenin was also estimated by the enzyme immunoassay method. Biotinylated actin (Bt-actin) was prepared and characterized as described previously (Choi *et al*, 1997). The recombinant human angiogenin was kindly provided by Dr. Chi-Bom Chae of Pohang Institute of Science and Technology, Pohang, Korea. Immunoplates were coated with angiogenin (0.1 μg/well), and blocked with 0.1% bovine serum albumin in the washing buffer (0.1% sodium azide, 0.05% Tween-20 in phosphate-buffered saline). Bt-actin was applied to the immunoplates at a final concentration of 1 nM with different amounts of AMBP15, and the immunoplates were incubated with shaking at room temperature for 1 h. The amount of bound Bt-actin was estimated with extravidin-conjugated alkaline phosphatase (Sigma, St. Louis, USA). The immunoplates were washed three times with washing buffer after each step.

Chorioallantoic membrane assay The chick chorioallantoic membrane assay was carried out by the method of Fett *et al.*

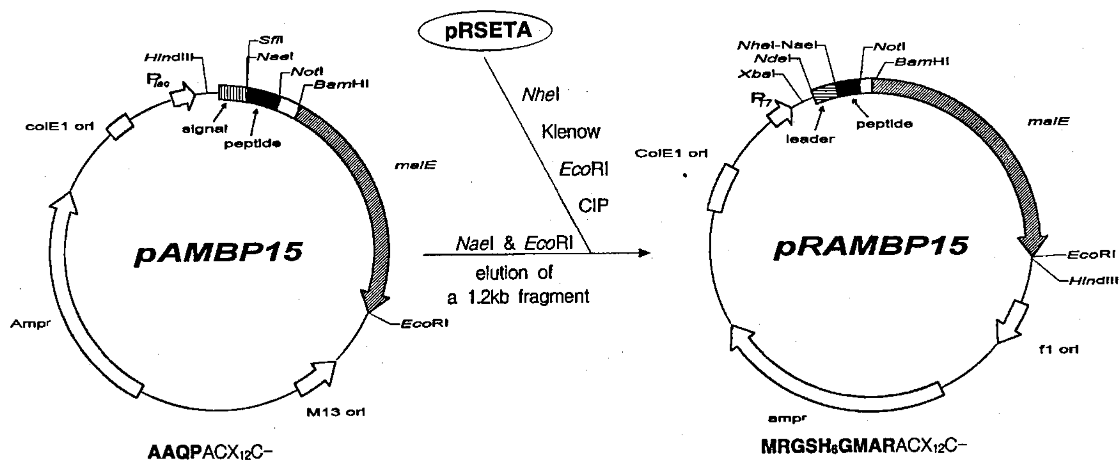

Fig. 1. Construction of a peptide expression vector pRAMBP15. pAMBP15 is a vector used in the previous work (Choi *et al.*, 1997). pAMBP was digested with *Nae*I and *Eco*RI and the resulting 1.2 kb fragment was inserted into the pRSETA vector by using the *Nhe*I and *Eco*RI sites. The expected N-terminal amino acid sequences of the two fusion proteins are shown below the vectors.

(1985). The fertilized eggs were purchased from Pulmuwon (Taejeon, Korea). The eggs were placed in a humidified incubator adjusted to 37°C (day 0). About 3 ml of albumin was removed from each egg at day 3. Windows were prepared at day 4. The protein samples were dialyzed against deionized water just prior to their use and applied to quarter pieces of sterile Thermanox coverslips (Nunc, Naperville, USA). The coverslips were dried under laminar flow conditions and applied to the eggs at day 9. The eggs were observed at day 11 with a microscope. Fat emulsion (10%) was injected before making the photographs.

Results and Discussion

Expression of AMBP15 In the previous work, the angiogenin binding peptide was expressed as a fusion protein with MBP by replacing gene III of the library vector with the *mal*E gene (Choi *et al.*, 1997). However, it was difficult to obtain the fusion protein in a large quantity, possibly because its expression requires the secretion machinery of the host cell. Therefore, a new expression vector, pRAMBP15, was constructed by inserting the peptide–MBP gene fragment into the pRSETA vector.

A significant expression of the fusion protein was observed by induction with 1 mM IPTG at 30°C (Fig. 2, lanes 1 and 2). The majority of the expressed protein was found in the soluble extract after lysis of the cell (Fig. 2, lane 4), and the protein was purified in a single step with an amylose column (Fig. 2, lane 5). The purified protein was found to be contaminated by a small amount of endogenous MBP. To find an optimal condition for the expression of the fusion protein, the incubation temperature and IPTG concentration were varied. The expression level of soluble AMBP15 was highest at 37°C with 0.2~1 mM IPTG as judged by SDS-PAGE and band intensity analysis (data not shown). In a later experiment, the fusion protein was routinely expressed at 37°C with 0.5 mM IPTG. The amount of the expressed protein was about 20 mg/l of culture at this optimal condition. We were able to obtain about 10 mg of purified AMBP15 per liter of culture. The yield was about 20-fold higher than that obtained previously, and the purified protein had less than 5% of endogenous MBP as concluded by the band intensity analysis. It is thought that the increased yield of the expressed protein is attributed to the strong T7 promoter and to the removal of the signal sequence of gene III.

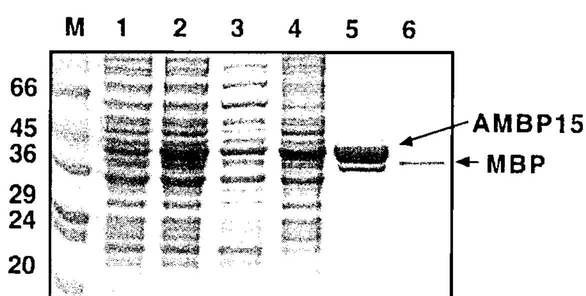

Fig. 2. Expression and purification of the AMBP15 fusion protein. The lanes 1 and 2 show the whole cell proteins of *E. coli* BL21 (DE3) pLysS containing pRAMBP15 before and after induction of protein expression with 1 mM IPTG at 30°C. After protein induction, the cells were homogenized and the clear supernatant (lane 4) was separated from insoluble cell debris (lane 3) by centrifugation. The soluble extract was applied to an amylose column and the bound proteins (lane 5) were collected as described in Materials and Methods. Lane 6 contained wild-type MBP purified from the same strain lacking pRAMBP15. The samples were electrophoresed in 10% denaturing polyacrylamide gel. The lane for the standard proteins is denoted as M and their molecular masses in kDa are shown in the left side of the gel.

MBP has been used as a fusion partner for the expression of peptides (Rondard *et al.*, 1997) as well as for proteins (Bedouelle and Duplay, 1988). The expressed fusion proteins remain soluble and active in most cases. In the phage-peptide library technique, selected peptides and their variants should be synthesized for the analysis of their activity, and our peptide–MBP fusion protein approach may be an economical alternative to the chemical synthesis of peptides. Moreover, the fusion proteins can be easily purified by an amylose affinity column and analyzed by the enzyme immunoassay method with an anti-MBP antibody. These features are very useful for the simultaneous preparation of multiple samples and for estimating the affinity of the peptides to their target molecules.

Enzyme immunoassay analysis of AMBP15 Angiogenin has a 35% amino acid sequence homology with RNase A. Moreover, the two proteins are closely related in their three-dimensional structure and enzymatic activity. However, AMBP15 showed only a basal level of binding to RNase A, while a saturating level of binding to angiogenin was observed in the same concentration range (Fig. 3). This shows a binding specificity of AMBP15 toward angiogenin.

Because the peptide carried by AMBP15 was already shown to possess substantial activity to inhibit the binding of Bt-actin to angiogenin (Choi *et al.*, 1997), we performed a binding assay of Bt-actin to angiogenin at a variable concentration of AMBP15 for the quantitative estimation of its inhibition activity. As shown in Fig. 4, AMBP15 inhibited the interaction between Bt-actin and angiogenin in a dose-dependent manner and a half-maximal inhibition was obtained at around 0.6 μM of AMBP15. The IC_{50} value is apparently lower than those of the peptides obtained by the hydropathic complementarity approach (Gho and Chae, 1997) or the peptide selected from a constrained octameric phage-peptide library (Gho *et al.*, 1997). However, it should be noted that the inhibition activity of the peptide in AMBP15 might be overestimated because of the fusion partner, MBP, as pointed out in the previous paper (Choi *et al.*, 1997). The affinity

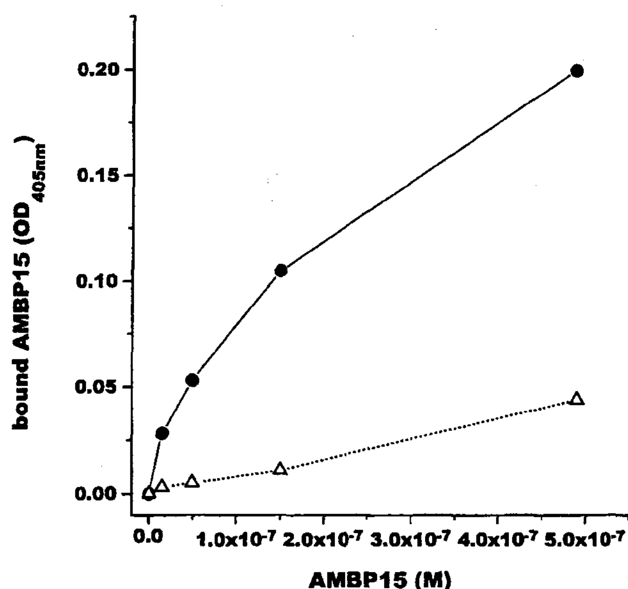

Fig. 3. Specific binding of AMBP15 to angiogenin. The binding of AMBP15 to angiogenin (solid line) or RNase A (dashed line) was estimated as described in the Materials and Methods.

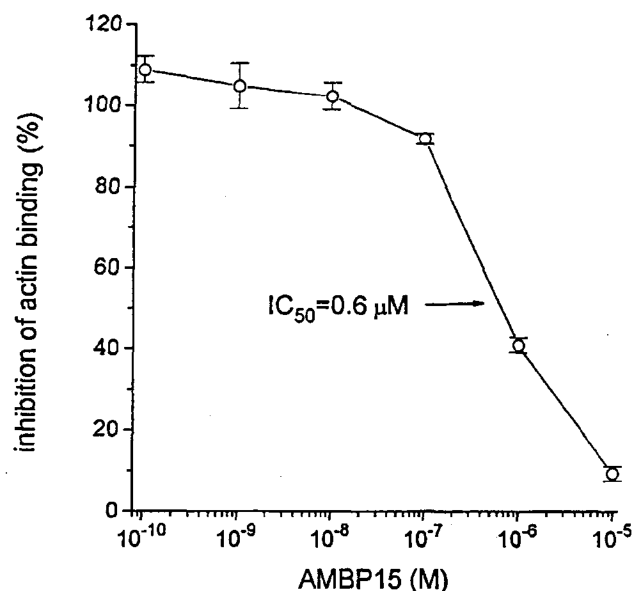

Fig. 4. Inhibition of the binding of Bt-actin to angiogenin by AMBP15. The effect of AMBP15 on the binding of Bt-actin to angiogenin was estimated as described in the Materials and Methods. Each point represents the average of three experiments. Error bars represent the standard error of each data point.

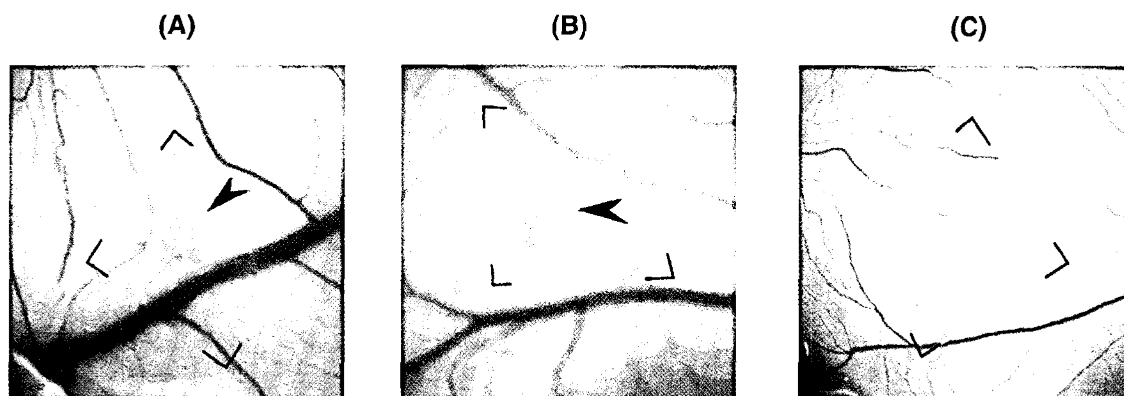

Fig. 5. Three kinds of responses in the chorioallantoic membrane assay. Chorioallantoic membrane assay was performed as described in the Materials and Methods. The location of the Thermanox coverslips is marked with brackets. (A) Positive responses were revealed by a formation of a clear spokewheel pattern. (B) The formation of a spot affecting nearby blood vessels was considered as a weak response. (C) Negative responses were clear by the absence of any spokewheel pattern or spot.

enhancement of the peptides by phage was also observed in the screening of peptides specific for the S-protein (Yu and Smith, 1996). Therefore, the chemically synthesized free peptides should be used for the precise comparison of their activity.

Anti-angiogenic activity of AMBP15 The binding of angiogenin to the cell surface actin is an essential step for the angiogenic process of stimulation (Hu *et al.*, 1993; Olson *et al.*, 1995). We performed chorioallantoic membrane assays to see if AMBP15 could inhibit the angiogenic activity. Three kinds of responses were observed: a strong response showing a typical spokewheel pattern (Fig. 5A), a weak response as revealed by the formation of a spot affecting nearby blood vessels (Fig. 5B), and no response (Fig. 5C). Only the formation of a clear spokewheel pattern was considered as a positive response. Angiogenic activity was almost reduced to a basal level with 20 pmol of AMBP15 (Table 1). Moreover, the positive responses obtained with the mixture (angiogenin and AMBP15) generally showed a less evident formation of new blood vessels when compared to the eggs

Table 1. Effect of AMBP15 on the angiogenic activity of angiogenin. The chorioallantoic membrane assay was performed as described in the Materials and Methods. The results are combined from two sets of assay, and each assay employed about 10 eggs per sample.

Sample	Eggs tested	Number of positive eggs	% positive response
Water	22	4	18
Angiogenin (1.4 pmol)	18	10	56
AMBP15 (20 pmol)	20	4	20
Angiogenin (1.4 pmol) +AMBP15 (20 pmol)	23	6	26

treated with angiogenin only. These results clearly show the anti-angiogenic activity of the AMBP15 fusion protein. Therefore, the AMBP15 fusion protein, or a synthetic peptide derived from AMBP15, may be used as an anti-angiogenic agent.

Acknowledgment This study was supported by NON DIRECTED RESEARCH FUND, Korea Research Foundation.

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