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(54) **Titre : COMPOSITION PHARMACEUTIQUE COMPRENANT L'ARGININE DEIMINASE DE LIAISON A L'ALBUMINE POUR LE  
TRAITEMENT CIBLE DU CANCER**  
(54) **Title: PHARMACEUTICAL COMPOSITION COMPRISING ALBUMIN-BINDING ARGININE DEIMINASE FOR CANCER  
TARGETING TREATMENT**

(57) **Abrégé/Abstract:**

The present invention provides a pharmaceutical composition containing albumin-binding arginine deiminase fusion protein (AAD) for treating cancer or other arginine-dependent diseases. The AAD fusion protein can be purified from both soluble and insoluble fractions of crude proteins, it binds to human serum albumin (HSA) and has its high activity with longer half life for efficient depletion of arginine in cancer cells. The specific activities of wild-type ADI and AAD in the present invention are 8.4 and 9.2 U/mg (at physiological pH 7.4), respectively. The AAD used in the present invention can be used in the treatment of various cancers (e.g. pancreatic cancer, leukemia, head and neck cancer, colorectal cancer, lung cancer, breast cancer, liver cancer, nasopharyngeal cancer, esophageal cancer, prostate cancer, stomach cancer & brain cancer) and curing arginine-dependent diseases. The composition can be used alone or in combination with at least one chemotherapeutic agent to give a synergistic effect on cancer treatment and/or inhibiting metastasis.



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(57) Abstract: The present invention provides a pharmaceutical composition containing albumin-binding arginine deiminase fusion protein (AAD) for treating cancer or other arginine-dependent diseases. The AAD fusion protein can be purified from both soluble and insoluble fractions of crude proteins, it binds to human serum albumin (HSA) and has its high activity with longer half life for efficient depletion of arginine in cancer cells. The specific activities of wild-type ADI and AAD in the present invention are 8.4 and 9.2 U/mg (at physiological pH 7.4), respectively. The AAD used in the present invention can be used in the treatment of various can-  
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WO 2014/138319 A8

## PHARMACEUTICAL COMPOSITION COMPRISING ALBUMIN-BINDING ARGININE DEIMINASE FOR CANCER TARGETING TREATMENT

### Cross-reference to Related Application

[0001] The present application claims benefit from US provisional patent application serial number 61/773,214 filed March 6, 2013 and US non-provisional patent application serial number 14/197,236 filed March 5, 2014, and the disclosures of which are incorporated herein by reference in its entirety.

### Technical Field

[0002] The present invention describes albumin-binding arginine deiminase (AAD) fusion protein that has been genetically modified to create a material having high activity and long *in vivo* half-life. The present invention further describes the designs for DNA and protein engineering for creating different AAD fusion proteins. The AAD fusion proteins can be isolated and purified from soluble fraction and insoluble fraction (inclusion bodies) of the crude proteins. The present invention further relates to albumin-binding arginine deiminase-containing pharmaceutical compositions for cancer targeting treatment and curing arginine-dependent diseases in humans and other animals.

### Background of the Invention

[0003] The incidence of pancreatic cancer, colon cancer, liver cancer, melanoma and cervical cancer in the worldwide population is increasing. Effective treatments for these diseases are urgently needed. In many types of cancer including leukemia, melanoma, pancreatic, colon, renal cell carcinoma, lung, prostate, breast, brain, cervical and liver cancers, the cancer cells are auxotrophic for arginine since they lack of expression of argininosuccinate synthetase (ASS), making these cancers excellent targets for arginine depletion therapy.

[0004] Arginine is a semi-essential amino acid for humans and other mammals. It can be synthesized from citrulline *via* a two step process catalyzed by the urea cycle enzymes

argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL). Arginine can be metabolized to ornithine by the enzyme arginase, and ornithine can be converted to citrulline by ornithine carbamoyltransferase (OTC) in the mitochondria. The citrulline can be utilized to synthesize arginine again. Normal cells usually do not require an exogenous supply of arginine for growth because of the abundant catalytic activity of ASS and ASL. In contrast, many types of cancers do not express ASS and therefore are auxotrophic for arginine. Their growth is dependent on arginine solely obtained from blood circulation. Therefore, targeting circulating arginine by using arginine-degrading enzymes is a feasible strategy to inhibit ASS-negative tumor growth [Feun et al., Curr. Pharm. Des. 14:1049-1057 (2008); Kuo et al., Oncotarget. 1:246-251 (2010)]

[0005] Arginine can be degraded by arginase, arginine decarboxylase, and arginine deiminase (ADI). Among them, arginine deiminase (ADI) appears to have the highest affinity for arginine (a low  $K_m$  value). ADI converts arginine to citrulline and ammonia, the metabolites of the urea cycle. Unfortunately, ADI can only be found in prokaryotes e.g. *Mycoplasma sp.* There are some problems associated with the isolation and purification of ADI from prokaryotes. ADI isolated from *Pseudomonas putida* fails to exhibit efficacy *in vivo* because of its low enzymatic activity in neutral pH. ADI produced from *Escherichia coli* is enzymatically inactive and subsequently requires multiple denaturation and renaturation process which raises the subsequent cost of production.

[0006] As the native ADI is found in microorganisms, it is antigenic and rapidly cleared from circulation in a patient. The native form of ADI is immunogenic upon injection into human circulation with a short half-life (~4 hours) and elicits neutralizing antibodies [Ensor et al., Cancer Res. 62:5443-5450 (2002); Izzo et al., J. Clin. Oncol. 22:1815-1822 (2004)]. These shortcomings can be remedied by pegylation. Among various forms of pegylated ADI, ADI bound with PEG (molecular weight 20,000) *via* succinimidyl succinate (ADI-PEG 20) has been found to be an efficacious formulation. However, the activity of ADI after pegylation is greatly decreased on the order of 50% [Ensor et al., Cancer Res. 62:5443-5450 (2002)]. The previous attempts to create pegylated ADI resulted in materials that are not homogenous (due to the random attachment of PEG on protein surface Lys residues) and also difficult to characterize and perform quality control during the manufacturing process. Also, PEG is very expensive, greatly increasing the production cost. After the intravenous injection of pegylated ADI *in vivo*, leakage

or detachment of free PEG is observed and the ADI (without PEG) can elicit the immunogenicity problem. Therefore, there is a need for improved cancer-treatment compositions, particularly, improved cancer-treatment compositions that have enhanced activity and *in vivo* half-life.

### Summary of the Invention

[0007] In the present invention, albumin-binding arginine deiminase (AAD) fusion protein has increased its activity and plasma half-life in order to efficiently deplete arginine in cancer cells. Native ADI may be found in microorganisms and is antigenic and rapidly cleared from circulation in a patient. The present invention constructs different AAD fusion proteins with one or two albumin-binding proteins to maintain high activity with longer *in vivo* half-life (at least 5 days of arginine depletion after one injection). In the present invention, the albumin binding protein in the AAD fusion protein product does not appear to influence its specific enzyme activity but instead appears to increase the circulating half-life. The specific activities of wild-type ADI and AAD fusion protein in the present invention are 8.4 and 9.2 U/mg (at physiological pH 7.4), respectively.

[0008] In its broadest sense, the present invention provides an albumin-binding arginine deiminase fusion protein comprising a first portion comprising one or two components selected from an albumin-binding domain, an albumin-binding peptide or an albumin-binding protein(s) fused to a second portion comprising arginine deiminase to form the albumin-binding arginine deiminase fusion protein such that the albumin-binding arginine deiminase fusion protein retains the activity of arginine deiminase and is also able to bind serum albumin.

[0009] The present invention further relates to albumin-binding arginine deiminase (AAD) fusion protein –containing pharmaceutical compositions for targeted cancer treatment in humans and other animals. The first aspect of the present invention is to construct the modified AAD fusion protein with high activity against cancer cells. The second aspect of the present invention is to purify AAD fusion protein with high purity from both soluble and insoluble fractions of the crude proteins. The third aspect of the present invention is to lengthen the half-life of AAD fusion protein as it can bind to albumin very well in the circulation. The fourth aspect of the present invention is to provide a method of using the AAD-containing pharmaceutical composition of the present invention for treating cancer by administering said composition to a

subject in need thereof suffering from various tumors, cancers or diseases associated with tumors or cancers or other arginine-dependent diseases.

[0010] The AAD fusion protein of the present invention is also modified to avoid dissociation into albumin-binding protein and ADI such that it becomes more stable and has a longer half-life in circulation. ADI is fused to an albumin-binding domain/peptide/protein in AAD fusion product to extend the plasma half-life and reduce the immunogenicity of the fusion product. Albumin binding domain (ABD) is a peptide that binds albumin in the blood. There are different variants of ABD showing different or improved human serum albumin (HSA) affinities. Different variants of ABD can be constructed and can be fused to ADI. Unlike the naturally occurring ADI, this longer half-life property facilitates the depletion of arginine efficiently in cancerous cells, cancer stem cells and/or cancer progenitor cells.

[0011] The pharmaceutical composition containing AAD fusion protein can be used for intravenous (i.v.) injection (for rapid-acting dosage of medication) and intramuscular (i.m.) injection (for fairly rapid-acting and long-lasting dosage of medication). The application of AAD fusion protein in the present invention can be used in the treatment of various cancers such as pancreatic cancer, leukemia, head and neck cancer, colorectal cancer, lung cancer, breast cancer, prostate cancer, cervical cancer, liver cancer, nasopharyngeal cancer, esophageal cancer and brain cancer. The present invention is directed to AAD fusion proteins, to methods of treating cancer, to methods of treating and/or inhibiting metastasis of cancerous tissue, and to methods of curing arginine-dependent diseases.

[0012] The method of the present invention also includes using a combination of different chemotherapeutic drugs and/or radiotherapy with the AAD fusion protein of the present invention to give a synergistic effect on cancer treatment.

### **Brief Description of the Drawings**

[0013] **FIG. 1** shows the design approach for construction of different AAD fusion proteins with one or two albumin-binding domain/peptide/protein(s) in three-dimensional structure. One or two albumin-binding domain/peptide/protein(s) can be fused to ADI to form the AAD fusion protein. The position of albumin-binding domain/peptide/protein is far from the ADI active site.

The albumin-binding domain/peptide/protein can be fused to the N-terminus or/and C-terminus of ADI. The structure in this figure is based on the *Mycoplasma arginini* ADI structure (Protein Data Bank: 1LXY). (A) Native ADI; (B) AAD fusion protein with two ABD or ABD1; (C) AAD fusion protein with one ABD or ABD1 at N-terminus; (D) AAD fusion protein with one ABD or ABD1 at C-terminus.

[0014] **FIG. 2** shows the sequence alignment for ADI in some bacterial species including *Mycoplasma arginini* (SEQ ID No. 23), *Lactococcus lactis* (SEQ ID No. 24), *Bacillus cereus* (SEQ ID No. 25) and *Bacillus licheniformis* (SEQ ID No. 26).

[0015] **FIG. 3** shows the designs and amino acid sequences for different AAD fusion proteins originated from *Mycoplasma arginini* (A to E) and AAD fusion protein originated from *Bacillus cereus* (F).

[0016] **FIG. 4** shows the creation of AAD fusion protein in two embodiments (A) and (B) by the use of intein-fusion proteins and expressed protein ligation (CBD, chitin binding domain) under the following schemes; (C) C-terminal fusion; (D) N-terminal fusion; (E) Intein-mediated protein ligation.

[0017] **FIG. 5** shows the plasmid map of the expression vector constructed for producing AAD fusion protein.

[0018] **FIG. 6** shows the (A) gene map, (B) nucleotide sequence (SEQ ID No. 44) and (C) amino acid sequence (SEQ ID No. 40) of His-ABD-PolyN-ADI. (ADI: the *Mycoplasma arginini* ADI)

[0019] **FIG. 7** shows the (A) gene map, (B) nucleotide sequence (SEQ ID No. 45) and (C) amino acid sequence (SEQ ID No. 41) of His-ABD-PolyN-bcADI. (bcADI, the *Bacillus cereus* ADI)

[0020] **FIG. 8** shows the expression and purification of AAD fusion protein: (A) AAD is ~90% soluble when expressed at 20°C (lanes 2 and 3) and ~90% insoluble (inclusion body) when expressed at 37°C (lanes 4 and 5); (B) The purified AAD fusion protein in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel: lane 1, purified AAD fusion protein (52.8 kDa); lane 2, molecular weight marker.

[0021] **FIG. 9** illustrates that AAD fusion protein depletes arginine efficiently and inhibits the growth of various types of human cancer cell lines in *in vitro* tissue culture studies, including human melanoma (A375), human colon carcinoma (HCT116), and human pancreatic cancer (PancI).

[0022] **FIG. 10** shows the albumin binding results of AAD fusion protein: (A) A non-denaturing native polyacrylamide gel (12%) showing the increase in the amount of HSA+AAD complex when the amount of AAD fusion protein (the amino acid sequence is shown in SEQ ID NO: 36; FIG. 3A) added increases. The mole ratios of human serum albumin (HSA): AAD in lanes 3-6 are 1:1, 1:2, 1:5, and 1:15, respectively. Lanes 1 and 2 represent HSA and AAD at 6 and 30 pmole, respectively; (B) In another experiment based on AAD fusion protein (SEQ ID NO: 40; FIG. 3E), an albumin: AAD ratio of 1:8 is sufficient to bind all the albumin present (lane 5).

[0023] **FIG. 11** is a graph showing the dose response of AAD fusion protein on plasma arginine levels in mice. A dose of 100  $\mu$ g of AAD is sufficient to deplete plasma arginine for at least 5 days.

## Definitions

[0024] The term “cancer stem cell” refers to the biologically distinct cell within the neoplastic clone that is capable of initiating and sustaining tumor growth *in vivo* (i.e. the cancer-initiating cell).

## Detailed Description of the Invention

[0025] Arginine is a semi-essential amino acid for humans and other mammals. It can be synthesized from citrulline *via* a two step process catalyzed by urea cycle enzymes argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL). Arginine can be metabolized to ornithine by the enzyme arginase, and ornithine can be converted to citrulline by ornithine carbamoyltransferase (OTC) in the mitochondria. The citrulline can be utilized to synthesize arginine again. Normal cells do not typically require an exogenous supply of arginine for growth because of the abundant catalytic activity of ASS and ASL. In contrast, many types of cancers do not express ASS and are therefore auxotrophic for arginine. Their growth is solely

dependent on arginine from circulation. Therefore, targeting circulating arginine by using arginine-degrading enzymes is a feasible strategy to inhibit ASS-negative tumor growth.

[0026] Arginine can be degraded by arginine deiminase (ADI). ADI converts arginine to citrulline and ammonia, the metabolites of the urea cycle. Unfortunately, ADI can only be found in prokaryotes e.g. *Mycoplasma sp.* There are many problems associated with the isolation and purification of arginine deiminase from prokaryotes. ADI isolated from *Pseudomonas putida* failed to exhibit efficacy *in vivo* because of its low enzymatic activity in neutral pH. ADI produced from *Escherichia coli* is enzymatically inactive and subsequently requires multiple denaturation and renaturation process which raised the subsequent cost of production. The plasma half-life of the native form of ADI is short (~4 hours) upon injection into human circulation [Ensor et al., Cancer Res. 62:5443-5450 (2002); Izzo et al., J. Clin. Oncol. 22:1815-1822 (2004)]. These shortcomings can be partially remedied by pegylation. Among various forms of pegylated ADI, ADI bound with PEG (molecular weight 20,000) *via* succinimidyl succinate (ADI-PEG 20) has been found to be an efficacious formulation. However, the activity of ADI after pegylation is greatly decreased (by ~50%) [Ensor et al., Cancer Res. 62:5443-5450 (2002); Wang et al., Bioconjug. Chem. 17:1447-1459 (2006)]. Also, the succinimidyl succinate PEG linker can easily be hydrolyzed and detached from the protein, causing immunogenic problems after a short period of use in the body. Therefore, there is a need for improved cancer-treatment compositions, particularly, improved cancer-treatment compositions with enhanced activity.

[0027] ADI isolated from *P. putida* failed to exhibit efficacy *in vivo* because it had little enzyme activity at a neutral pH and was rapidly cleared from the circulation of experimental animals. ADI derived from *Mycoplasma arginini* is described, for example, by Takaku et al, Int. J. Cancer, 51:244-249 (1992), and U.S. Pat. No. 5,474,928. However, a problem associated with the therapeutic use of such a heterologous protein is its antigenicity. The chemical modification of ADI from *Mycoplasma arginini*, *via* a cyanuric chloride linking group, with polyethylene glycol (PEG) was described by Takaku et al., Jpn. J. Cancer Res., 84:1195-1200 (1993). However, the modified protein was toxic when metabolized due to the release of cyanide from the cyanuric chloride linking group. In contrast, even for the ADI-PEG20, the PEG linker can easily be hydrolyzed and detached from the protein, causing immunogenic problems after a short period of

use in the body. Therefore, there is a need for compositions which degrade non-essential amino acids and which do not have the problems associated with the prior art.

[0028] In many types of cancer including melanoma, pancreatic, colon, leukemia, breast, prostate, renal cell carcinoma and liver cancers, cancer cells are auxotrophic for arginine since they lack of expression of argininosuccinate synthetase (ASS), making them excellent targets for arginine depletion therapy. In this invention, albumin-binding arginine deiminase (AAD) fusion proteins have high activity with long half-lives for efficient depletion of arginine in cancer cells.

[0029] The size of the monomer for ADI is on the order of 45 kDa and it exists as dimer (on the order of 90 kDa) [Das et al., Structure. 12:657-667 (2004)]. A design for construction of an AAD fusion protein is shown in FIG. 1. One or two albumin-binding domain/peptide/protein(s) with or without linker(s), SEQ ID NO: 46-49, are fused to ADI to form the AAD fusion protein. It is noteworthy that the selection of one or two particular albumin-binding domain/peptide/protein(s) can be made depending upon the type of cancer tissue to be targeted, the desired size and half-life of the resulting fusion protein, and whether a domain or entire protein is selected. Further, the selected albumin-binding material may be the same or different. That is, a protein and a peptide can be fused, two proteins, two domains, a domain and a protein, etc., as long as the resultant molecule retains the activity of the ADI and is also able to bind serum albumin with neither function of one portion of the fusion protein being interfered with by the other portion of the fusion protein. The position of the albumin-binding domain/peptide/protein is far from the active site. The albumin-binding domain/peptide/protein can be fused to the N-terminus or/and C-terminus of ADI. There are different variants of ABD showing different or improved human serum albumin (HSA) affinities. Different variants of ABD can be constructed and can be fused to ADI. Some micro-organisms endowed with ADI (for example *Pseudomonas* sp) cannot be used, due to their potential pathogenicity and pyrogenicity. The source of ADI can be from, but not limited to, different microorganisms, e.g. *Mycoplasma* (e.g. *Mycoplasma arginini*, *Mycoplasma arthritidis*, *Mycoplasma hominis*), *Lactococcus* (e.g. *Lactococcus lactis*), *Pseudomonas* (e.g. *Pseudomonas plecoglossicida*, *Pseudomonas putida*, *Pseudomonas aeruginosa*), *Streptococcus* (e.g. *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus pneumoniae*), *Escherichia*, *Mycobacterium* (e.g. *Mycobacterium tuberculosis*) and *Bacillus* (e.g. *Bacillus licheniformis*, *Bacillus cereus*). It is preferred that ADI is cloned from

*Mycoplasma arginini*, *Lactococcus lactis*, *Bacillus licheniformis*, *Bacillus cereus*, or any combination thereof. Their amino acid sequences with SEQ ID (SEQ ID NO: 23-35) and the sequence alignment for some of the amino acid sequences in FIG. 2 are disclosed herein and also in the literature [Das et al., Structure. 12:657-667 (2004); Wang et al., Bioconjug. Chem. 17:1447-1459 (2006); Ni et al., Appl. Microbiol. Biotechnol. 90:193-201 (2011)].

[0030] The design and amino acid sequence for (A) native *Mycoplasma arginini* ADI protein (SEQ ID NO: 23), (B) different AAD fusion proteins originated from the *Mycoplasma arginini* ADI (SEQ ID NO: 36-40) and (C) AAD fusion protein originated from the *Bacillus cereus* ADI (SEQ ID NO: 41) are shown in FIG. 3. Different AAD fusion proteins are successfully constructed. A linker is inserted between the albumin-binding protein and ADI in the AAD fusion protein in these embodiments.

[0031] On the other hand, a novel AAD fusion protein is also created by the use of intein-fusion proteins and expressed protein ligation (FIG. 4). The novel AAD fusion protein can be formed (1) by reacting the ADI having a N-terminal cysteine residue with a reactive thioester at C-terminus of the ABD, or (2) by reacting the ABD having a N-terminal cysteine residue with a reactive thioester at C-terminus of the ADI so that the ADI and the ABD are linked by a covalent bond. In FIG. 4E, ADI with N-terminal cysteine residue reacts with reactive thioester at the C-terminus of ABD. The thioester tag at the C-terminus of ABD, and an  $\alpha$ -cysteine at the N-terminus of ADI are required to facilitate protein ligation. These fragments are produced using a pTWIN1 vector (New England Biolabs) according to the manufacturer's manual. In particular, the gene coding for the ABD-Intein-CBD fusion protein is synthesized and it is cloned into the vector under the control of T7 promoter for expression in *E. coli* (FIG. 4C). The ABD-Intein-CBD fusion protein produced binds to chitin in a column. The amino acid sequence of ABD-Intein-CBD (SEQ ID NO: 42) is shown in FIG. 4A. After thiol-inducible cleavage and elution from the column, the ABD with reactive thioester at its C-terminus is obtained (FIG. 4C). On the other hand, the gene coding for the CBD-Intein-ADI fusion protein is synthesized and cloned into the vector under the control of the T7 promoter for expression in *E. coli* (FIG. 4D). The CBD-Intein-ADI fusion protein produced binds to chitin in a column. The amino acid sequence of the CBD-Intein-ADI (SEQ ID NO: 43) is shown in FIG. 4B. After cleavage at pH 7 and 25°C, and elution from the column, the ADI with  $\alpha$ -cysteine at its N-terminus is obtained (FIG. 4D). Finally, the AAD fusion protein is produced by the protein ligation reaction as shown in FIG. 4E.

[0032] Importantly, AAD fusion proteins can be produced and purified in a convenient manner. For example, an AAD fusion protein is successfully expressed and purified from *E. coli* both in soluble fraction and insoluble fraction, and this result is shown in FIG. 8. Furthermore, FIG. 8 shows the purified AAD fusion protein analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The size of the purified AAD fusion protein is determined as 52.8 kDa.

[0033] The pharmaceutical composition of the present invention contains AAD fusion protein with high activity for depleting arginine in tumor cells for cancer treatment. The specific activity of the purified AAD fusion protein is found to be similar to that of the wild-type ADI.  $IC_{50}$  is the half maximal inhibitory concentration, that is, it represents the concentration of AAD fusion protein that is required for 50% inhibition of a cancer cell line. The  $IC_{50}$  is a measure of the effectiveness of a drug. The  $IC_{50}$  of AAD fusion protein (amino acid sequence is shown in SEQ ID NO: 40, FIG. 3E) for different cancer cell lines (human melanoma, A375 & SK-mel-28; human colon carcinoma, HCT116; human pancreatic cancer, PancI; human liver cancer, Sk-hep1; human cervical cancer, C-33A) is shown in TABLE 1. The *in vitro* efficacy of AAD fusion protein on different cancer cell lines is demonstrated in FIG. 9. It illustrates that AAD fusion protein can kill many cancer types, including human melanoma, human colon carcinoma and pancreatic cancer cell lines.

[0034] **TABLE 1**

| Cancer cell line                | $IC_{50}$ of AAD ( $\mu\text{g/ml}$ ) |
|---------------------------------|---------------------------------------|
| A375 (human melanoma)           | 0.104                                 |
| SK-mel-28 (human melanoma)      | 1.92                                  |
| PancI (human pancreatic cancer) | 1                                     |
| Sk-hep1 (human liver cancer)    | 10                                    |

|                                |       |
|--------------------------------|-------|
| C-33A (human cervical cancer)  | 0.063 |
| HCT116 (human colon carcinoma) | 1.30  |

[0035] For the albumin binding study, we have demonstrated successfully that the engineered AAD fusion protein can bind to human serum albumin (HSA). FIG. 10 shows that the AAD fusion protein (amino acid sequence is shown in SEQ ID NO: 40, FIG. 3E) binds to HSA readily. At a mole ratio of 1:5 or 1:15, the formation of the HSA-AAD complex forms according to the construct of FIG. 1 using the linker molecule design. It is expected that the circulating half-life of AAD fusion protein in the blood is increased by the non-covalent HSA-AAD complex formation. Therefore, a long-lasting version of AAD fusion protein has been successfully created.

[0036] No commercial products show high efficacy when compared to the AAD fusion protein-containing pharmaceutical composition prepared in this invention. For uses in cancer treatment, the AAD fusion protein-containing pharmaceutical composition of the present invention serves as an anti-cancer agent to deplete the arginine in tumor tissues. AAD fusion protein is a good candidate to be used in combination with other molecular targeting or cytotoxic agents.

## Examples

[0037] The following examples are provided by way of describing specific embodiments of this invention without intending to limit the scope of this invention in any way.

[0038] Several of the Examples below relate to methods of making an albumin-binding arginine deiminase fusion protein. Various techniques can be used including cloning and intein-mediated protein ligation. As used herein, the term “cloning” is broadly used and comprises constructing a fusion gene coding for the albumin-binding arginine deiminase fusion protein, inserting the fusion gene into a vector, inserting the vector into a host organism and expressing a protein that includes an albumin-binding arginine deiminase fusion protein. Numerous variants on this technique can be performed and still fall within the cloning contemplated by the present invention.

[0039] **Example 1**[0040] **Construction of the gene coding for albumin-binding domain/peptide/protein (ABD)**

[0041] The gene coding for ABD is constructed by two rounds of PCR. In the first round, the PCR reaction mixture (total volume of 25  $\mu$ l) contains the following materials:

1 x iProof PCR buffer (Bio-Rad)

50  $\mu$ M dNTP mixture

0.5 unit of iProof DNA Polymerase (Bio-Rad)

10 nM of each of the following oligos

*ABD-F1 forward primer* (SEQ ID NO: 01):

5' -CATGATGCGAATTCCTTAGCTGAAGCTAAAGTCTTAGCTAACAGAGAACT-3'

*ABD-R2 reverse primer* (SEQ ID NO: 02):

5' -TAGTCACTTACTCCATATTTGTCAAGTTCTCTGTTAGCTAAGACTTTAGC-3'

*ABD-F3 forward primer* (SEQ ID NO: 03):

5' -GAACTTGACAAATATGGAGTAAGTGACTATTACAAGAACCTAATCAACAA-3'

*ABD-R4 reverse primer* (SEQ ID NO: 04):

5' -TACACCTTCAACAGTTTTGGCATTGTTGATTAGGTTCTTGTAATAGTCAC-3'

*ABD-F5 forward primer* (SEQ ID NO: 05):

5' -GCCAAAAGTGTGGAAGGTGTAAAAGCACTGATAGATGAAATTTTAGCTGC-3'

*ABD-R6 reverse primer* (SEQ ID NO: 06):

5' -AGCTACGATAAGCTTAAGGTAATGCAGCTAAAATTTTCATCTATCAGTG-3'

The following PCR program is used:

98°C 30 s; 20 cycles of {98°C 10 s, 50°C 20 s, 72°C 20 s}

[0042] In the second round of PCR, the PCR mixture (total volume of 50  $\mu$ l) contains the following materials:

1 x iProof PCR buffer (Bio-Rad);

50  $\mu$ M dNTP mixture;

1  $\mu$ l of PCR reactant as DNA template from the first round;

1 unit of iProof DNA Polymerase (Bio-Rad);

200 nM of each of the following oligos:

*ABD-F7 forward primer* (SEQ ID NO: 07):

5' -CATGATGCGAATTCCTTAGCTGAAGCTAAAGTCTTAGCTAACAGAGAACT-3'

*ABD-R8 reverse primer* (SEQ ID NO: 08):

5' -AGCTACGATAAGCTTAAGGTAATGCAGCTAAAATTTTCATCTATCAGTG-3'

The following PCR program is used:

98°C 30 s; 35 cycles of {98°C 10 s, 60°C 20 s, 72°C 20 s}; 72°C 5 min

[0043] A PCR product containing the DNA sequence of ABD (169 bp) is obtained and purified by Qiagen DNA Gel Extraction Kit for cloning purpose.

#### [0044] **Example 2A**

#### [0045] **Construction of the fusion gene coding for the AAD fusion protein**

[0046] In the first PCR, the PCR mixture (total volume of 50  $\mu$ l) contains the following materials:

1 x iProof PCR buffer (Bio-Rad);

50  $\mu$ M dNTP mixture;

25 ng of *Mycoplasma arginini* genomic DNA;

1 unit of iProof DNA Polymerase (Bio-Rad);

200 nM of each of the following oligos:

*ADINde-F forward primer* (SEQ ID NO: 09):

5' -ATCGATCGATGTCTGTATTTGACAGTAAATTAAAGG-3'

*ADIhis-R reverse primer* (SEQ ID NO: 10):

5' -AGCTAAGGAATTCGCATCATGATGGTGATGGTGGTGGCTACCCCACTTAAC-3'

The following PCR program is used:

98°C 1 min; 35 cycles of {98°C 10s, 50°C 20s, 72°C 40s}; 72°C 5 min

A PCR product of 1280 bp long is obtained and purified by Qiagen DNA Gel Extraction Kit. After that, the second PCR is performed. The PCR mixture (total volume of 50  $\mu$ l) contains the following materials:

1 x iProof PCR buffer (Bio-Rad);

50  $\mu$ M dNTP mixture;

10 ng of the 1280 bp PCR product;

10 ng of the 169 bp PCR product;

1 unit of iProof DNA Polymerase (Bio-Rad);

200 nM of each of the following oligos:

*ADINde-F forward primer* (SEQ ID NO: 11):

5' -ATCGATCGATGTCTGTATTTGACAGTAAATTTAAAGG-3'

*ABD-R10 reverse primer* (SEQ ID NO: 12):

5' -AGCTACGATAAGCTTAAGGTAATGCAGCTAAAATTTTCATCTATCAGTG-3'

The following PCR program is used:

98°C 1 min; 35 cycles of {98°C 10s, 50°C 20s, 72°C 45s}; 72°C 5 min

[0047] A PCR product of 1428 bp is obtained and purified by Qiagen DNA Gel Extraction Kit. Then it is digested with restriction enzymes NdeI and HindIII, and ligated to plasmid pREST A (Invitrogen) that is predigested with the same enzymes. The ligation product is then transformed into *E. coli* BL21 (DE3) cells. The sequence of the constructed fusion gene is confirmed by DNA sequencing.

#### [0048] **Example 2B**

#### [0049] **Cloning of His-ABD-PolyN-ADI**

[0050] The construction of **His-ABD-PolyN-ADI** (SEQ ID NO: 40, in FIG. 3E) is done by two steps of overlapping PCR, the PCR fragment obtained from the last step is inserted into the vector pET3a between the NdeI and BamHI sites. The gene map, nucleotide sequence and amino acid sequence of **His-ABD-PolyN-ADI** are shown in FIG. 6.

Primers involved in construction of **His-ABD-PolyN-ADI**:

*hisABDNde-F forward primer* (SEQ ID NO: 13):

5' -GGAGATATACATATGCATCATCACCATCACCATGATGAAGCCGTGGATG-3'

*ABDnn-R1 reverse primer* (SEQ ID NO: 14):

5' - TTGTTATTATTGTTGTTACTACCCGAAGGTAATGCAGCTAAAATTTTCATC - 3'

*ABDn-R2 reverse primer* (SEQ ID NO: 15):

5' - AGAACCGCCGCTACCATTGTTATTATTGTTGTTACTACCCGA - 3'

*ADIn-F forward primer* (SEQ ID NO: 16):

5' - AATAATAACAATGGTAGCGGCGGTTCTGTATTTGACAGTAAATTTAAAGG - 3'

*ADIBam-R reverse primer* (SEQ ID NO: 17):

5' - TAGATCAATGGATCCTTACCACTTAACATCTTTACGTGATAAAG - 3'

[0051] In the first round of PCR, 50 µl of reaction volume containing the known concentration of components are prepared in two PCR tubes. In each of the tubes, dNTP, iProof buffer (BIO-RAD), iProof DNA polymerase (BIO-RAD), primers and DNA template are mixed and added up to 50 µl by ddH<sub>2</sub>O. The DNA template used in the reaction is a pET3a vector containing the gene of ADI from *Mycoplasma arginini* with a removal of an internal NdeI site mutation without altering the protein sequence of the ADI gene.

[0052] The two reaction tubes contain the primer mixtures of (A) 10 pmol *hisABDNde-F* (SEQ ID NO: 13), 0.5 pmol *ABDnn-R1* (SEQ ID NO: 14) and 10 pmol *ABDn-R2* (SEQ ID NO: 15); and (B) 10 pmol *ADIn-F* (SEQ ID NO: 16) and 10 pmol *ADIBam-R* (SEQ ID NO: 17), respectively.

[0053] The PCR program is set according to the recommended steps in the manual with an annealing and extension temperature (time) at 50 °C (20 s) and 72 °C (40 s), respectively. The

two products generated by PCR with the size of 237 bp and 1278 bp. The products are extracted and applied as template for the next round of PCR.

[0054] In the second overlapping step, the reaction mixture is prepared in a similar way to the first round except the template used was the mixture of 1 pmol of the 237 bp PCR product and 1 pmol of the 1278 bp PCR product from the first round PCR. Primers used are changed to 10 pmol *hisABDNde-F* (SEQ ID NO: 13) and 10 pmol *ADIBam-R* (SEQ ID NO: 17).

[0055] The annealing and extension temperature (time) are 50 °C (20 s) and 72 °C (60 s), respectively. A PCR product with the size of 1484 bp is generated from the reaction. The PCR product is purified and digested with NdeI and BamHI and then ligated into the pre-digested pET3a plasmid. The ligated product is then transformed into *E. coli* BL21 (DE3) for the production of recombinant protein.

#### [0056] **Example 2C**

##### [0057] **Cloning of His-ABD-PolyN-bcADI**

[0058] The construction of **His-ABD-PolyN-bcADI** (SEQ ID NO: 41, in FIG. 3F) is done by two steps of overlapping PCR, the PCR fragment obtained from the last step is inserted into the vector pET3a between the NdeI and BamHI sites. The gene map, nucleotide sequence and amino acid sequence of **His-ABD-PolyN-bcADI** are shown in FIG. 7.

Primers involved in construction of **His-ABD-PolyN-bcADI**:

*hisABDNde-F2 forward primer* (SEQ ID NO: 18):

5' -GGAGATATACATATGCATCATCACCATCACCATGATGAAGCCGTGGATG-3'

*bcABDnn-R1 reverse primer* (SEQ ID NO: 19):

5' -TTGTTATTATTGTTGTTACTACCCGAAGGTAATGCAGCTAAAATTTCATC-3'

*bcABDn-R2 reverse primer* (SEQ ID NO: 20):

5' -TTTACCGCCGCTACCATTGTTATTATTGTTGTTACTACCCGA-3'

*bcADIn-F forward primer* (SEQ ID NO: 21):

5' -AATAATAACAATGGTAGCGGCGGTAAACATCCGATACATGTTACTTCAGA-3'

*bcADIBam-R reverse primer* (SEQ ID NO: 22):

5' -TAGATCAATGGATCCCTAAATATCTTTACGAACAATTGGCATAC-3'

[0059] In the first round of PCR, 50 µl of reaction volume containing the known concentration of components are prepared in two PCR tubes. In each of the tubes, dNTP, iProof buffer (BIO-RAD), iProof DNA polymerase (BIO-RAD), primers and DNA template are mixed and added up to 50 µl by ddH<sub>2</sub>O. The DNA template used in the reaction is a pET3a vector containing the gene of ADI from *Bacillus cereus* with a removal of an internal NdeI site mutation without altering the protein sequence of the ADI gene.

[0060] The two reaction tubes contain the primer mixtures of (A) 10 pmol *hisABDNde-F2* (SEQ ID NO: 18), 0.5 pmol *bcABDnn-R1* (SEQ ID NO: 19) and 10 pmol *bcABDn-R2* (SEQ ID NO: 20); and (B) 10 pmol *bcADIn-F* (SEQ ID NO: 21) and 10 pmol *bcADIBam-R* (SEQ ID NO: 22), respectively. The PCR program is set according to the recommended steps in the manual with an annealing and extension temperature (time) at 50 °C (20 s) and 72 °C (40 s), respectively. The two products are generated by PCR with the size of 237 bp and 1250 bp. The products are extracted and applied as template for the next round of PCR.

[0061] In the second overlapping step, the reaction mixture is prepared in a similar way to the first round except the template used is the mixture of 1 pmol of the 237 bp PCR product and 1 pmol of the 1250 bp PCR product from the first round PCR. Primers used are changed to 10 pmol *hisABDNde-F2* (SEQ ID NO: 18) and 10 pmol *bcADIBam-R* (SEQ ID NO: 22).

[0062] The annealing and extension temperature (time) are 50 °C (20 s) and 72 °C (60 s), respectively. A PCR product with the size of 1512 bp is generated from the reaction. The PCR product is purified and digested with NdeI and BamHI and then ligated into the pre-digested pET3a plasmid. The ligated product is then transformed into *E. coli* BL21 (DE3) for the production of recombinant protein.

### [0063] **Example 3**

#### [0064] **Expression and purification of the AAD fusion protein**

[0065] For preparing the seed culture, the strain *E. coli* BL21(DE3) carrying the plasmid encoding the AAD fusion protein (FIG. 5) is cultured in 5 ml of 2xTY medium, 30°C, 250 rpm, overnight. The overnight seed culture (2.5 ml) is added to 250 ml of 2xTY, 37°C, 250 rpm, 2.5 h (until OD<sub>600</sub> ≈ 0.6-0.7). When the OD<sub>600</sub> reached, IPTG is added to the culture (0.2 mM final concentration). The growth is continued for 22 more hours at 20°C and then the cells are collected by centrifugation. The cell pellet is resuspended in 25 ml of 10 mM sodium phosphate buffer, pH 7.4. The cells are lysed by sonication. The soluble portion is collected after centrifugation. The fusion protein (containing a His tag) is then purified by nickel affinity chromatography. TABLE 2 shows that cultivation temperature is an important factor in affecting the solubility of AAD fusion protein (amino acid sequence is shown in SEQ ID NO: 40, FIG. 3E) obtained from the expression host.

[0066] For isolating the soluble fraction of AAD fusion protein, the cell pellet is resuspended in 25 ml of 10 mM sodium phosphate buffer, pH 7.4. The cells are lysed by sonication. The soluble portion is collected after centrifugation. The AAD fusion protein (contains a His tag) is then purified by nickel affinity chromatography.

[0067] For isolating the insoluble fraction of AAD fusion protein, the cell pellet is resuspended in 25 ml of 20 mM Tris-HCl, pH 7.4, 1% Triton X-100. The cells are lysed by sonication. The insoluble portion (inclusion bodies) is collected by centrifugation. The protein is unfolded by resuspending in 10 ml of 20 mM Tris-HCl, pH 7.4, 6 M Guanidine HCl, and vortexed until it becomes soluble. The protein is refolded by adding the unfolded protein solution drop by drop into a fast stirring solution of 100 ml of 20 mM Sodium phosphate buffer, pH 7.4. The insoluble

materials are removed by centrifugation. Salting out of the protein is performed by adding solid ammonium sulphate powder into the supernatant to achieve 70% saturation. The insoluble portion is collected by centrifugation and it is resuspended in 10 ml of 20 mM sodium phosphate buffer. The AAD fusion protein (contains a His tag) is then purified by nickel affinity chromatography.

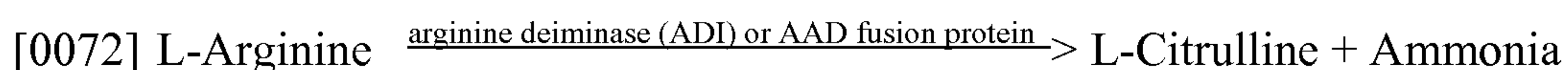
[0068] **TABLE 2**

| <b>AAD</b>                                   | <b>1</b>    | <b>2</b>    | <b>3</b>           |
|----------------------------------------------|-------------|-------------|--------------------|
| <b>Cultivation temperature (°C)</b>          | 30          | 20          | 37                 |
| <b>Yield (mg) / 250ml culture</b>            | ~0.66       | ~12.0       | ~7.0               |
| <b>solubility</b>                            | 50% soluble | 90% soluble | 90% inclusion body |
| <b>IC<sub>50</sub> (µg/ml) on A375 cells</b> | 0.10        | 0.68        | 0.23               |

[0069] **Example 4**

[0070] **Enzyme activity assay and Enzyme kinetics for AAD fusion protein**

[0071] To determine the enzyme activity for wild-type ADI and AAD fusion protein in the present invention, the diacetyl monoxime (DAM) - thiosemicarbazide (TSC) assay for citrulline detection is used. The reaction is shown below.



[0073] This assay is run by adding sample to a color reagent, which is made by mixing acidic ferric chloride solution with DAM-TSC solution. Briefly, enzyme is incubated with 20 mM arginine, 10 mM sodium phosphate pH 7.4 for 5 min at 37°C. The reaction mixture is heated at 100°C for 5 min to develop the color and read at 540 nm (light path = 1 cm). A standard curve is constructed using various concentrations of citrulline. One unit of the ADI native enzyme is the amount of enzyme activity that converts 1 µmol of arginine to 1 µmol of citrulline per minute at

37°C under the assay conditions. The specific activities of wild-type ADI and AAD fusion protein in the present invention are 8.4 and 9.2 U/mg (at pH 7.4, physiological pH) respectively. The specific activities for wild-type ADI and AAD fusion protein at different pH range (from pH 5.5 to 9.5) are also determined, and the optimum pH is at 6.5. Therefore, the results indicate that AAD fusion protein depletes arginine efficiently, as the fusion with albumin-binding protein does not affect enzyme activity of ADI.

[0074] The Michaelis constant  $K_m$  is the substrate concentration at which the reaction rate is at half-maximum, and is an inverse measure of the substrate's affinity for the enzyme. A small  $K_m$  indicates high affinity for the substrate, and it means that the rate will approach the maximum reaction rate more quickly. For determination of the enzyme kinetics or  $K_m$  value, the activity of wild-type ADI and AAD fusion protein are measured under different concentration of substrate arginine (2000  $\mu$ M, 1000  $\mu$ M, 500  $\mu$ M, 250  $\mu$ M, 125  $\mu$ M, 62.5  $\mu$ M) at pH 7.4. The measured  $K_m$  values of the AAD fusion protein shown in FIG. 3E (SEQ ID NO: 40, ADI protein is originated from *Mycoplasma arginini*) and AAD fusion protein shown in FIG. 3F (SEQ ID NO: 41, ADI protein is originated from *Bacillus cereus*) are 0.0041 mM and 0.132 mM respectively. The results suggest that the fusion to ABD did not affect the binding affinity of the different AAD fusion proteins to arginine.

#### [0075] **Example 5**

##### [0076] **Cell proliferation assay and *in vitro* efficacy of AAD fusion protein on cancer cell lines**

[0077] Culture medium DMEM is used to grow the human melanoma A375 & SK-mel-28, human pancreatic cancer PancI and human cervical cancer C-33A cell lines. The EMEM medium is used to culture the SK-hep 1 liver cancer and C-33A cervical cancer cell line. Cancer cells ( $2-5 \times 10^3$ ) in 100  $\mu$ l culture medium are seeded to the wells of 96-well plates and incubated for 24 h. The culture medium is replaced with medium containing different concentrations of AAD fusion protein. The plates are incubated for an additional 3 days at 37°C in an atmosphere of 95% air/5% CO<sub>2</sub>. MTT assay is performed to estimate the number of viable cells in the culture according to manufacturer's instructions. The amount of enzyme needed to achieve 50% inhibition of cell growth is defined as IC<sub>50</sub>.

[0078] As shown in TABLE 1 and FIG. 9, the results indicate that AAD fusion protein depletes arginine efficiently and inhibits the growth of various types of human cancer cell lines in *in vitro* tissue culture studies. For example, human melanoma, human colon carcinoma, human pancreatic cancer, human liver cancer and human cervical cancer, all have low values of IC<sub>50</sub> (see TABLE 1), as these cancer types are all inhibited by AAD fusion protein readily. As predicted, AAD fusion protein would inhibit all cancer types that are arginine-dependent (for example, the ASS-negative cancers).

[0079] **Example 6**

[0080] ***In vivo* half-life determination of AAD fusion protein**

[0081] Balb/c mice (5-7 weeks) are used in this study and they are allowed to acclimatize for a week before the experiment. Mice (n=3) are separated into four groups and injected with 0, 100, 500 or 1000 µg of AAD fusion protein (SEQ ID NO: 40, FIG. 3E) in 100 µl PBS intraperitoneally, respectively. Blood of each mouse is collected at 0 h and Day 1-7. Sera are obtained after centrifugation. The sera are then deproteinised and analyzed by amino acid analyzer for arginine.

[0082] As shown in FIG. 11, AAD fusion protein (SEQ ID NO: 40, FIG. 3E), even at the lowest dosage of 100 µg, depletes plasma arginine efficiently at Day 1, 3 and 5, suggesting that AAD can deplete arginine *in vivo* efficiently for at least 5 days. The arginine level returns to normal gradually at Day 6 and Day 7 in all treatment groups.

[0083] **Example 7**

[0084] ***In vivo* efficacy of AAD fusion protein on cancer cell xenografts**

[0085] Nude balb/c mice (5-7 weeks) are used in this study and they are allowed to acclimatize for a week before the experiment. Mice are inoculated subcutaneously with 2x10<sup>6</sup> cancer cells in 100 µl of fresh culture medium. Ten days later, the mice are randomly separated into control and treatment group. Control group receives 100 µl PBS and treatment group receives 100 µl AAD fusion protein intraperitoneally weekly. Tumor size is measured by caliper and tumor volume is

calculated using formula:  $(\text{length} \times \text{width}^2)/2$ . Blood draw are obtained at Day 5 after each treatment for plasma measurement of arginine.

## Replacement Sheets

## WHAT IS CLAIMED IS:

1. An albumin-binding arginine deiminase fusion protein comprising a first portion comprising one or two components selected from an albumin-binding domain, an albumin-binding peptide or an albumin-binding protein(s) fused to a second portion comprising arginine deiminase to form the albumin-binding arginine deiminase fusion protein, and one or more linker molecules; the first portion being positioned far from active site of the second portion by said linker molecule such that the albumin-binding arginine deiminase fusion protein retains the activity of arginine deiminase and binds serum albumin with neither function of one portion of the fusion protein being interfered with by the other portion of the fusion protein, wherein the albumin-binding arginine deiminase fusion protein comprises an amino acid sequence selected from SEQ ID NO: 36, 37, 38, 39, 40, or 41.
2. An albumin-binding arginine deiminase fusion protein according to claim 1 wherein the two components are the same.
3. An albumin-binding arginine deiminase fusion protein according to claim 1 wherein the two components are different.
4. An albumin-binding arginine deiminase fusion protein according to claim 1 wherein the albumin-binding domain is SEQ ID NO. 46, 47, 48, 49 or an active portion thereof.
5. An albumin-binding arginine deiminase fusion protein according to claim 1 wherein the albumin binding peptide is SEQ ID NO. 46, 47, 48, 49 or an active portion thereof.
6. An albumin-binding arginine deiminase fusion protein according to claim 1 wherein the albumin binding protein is SEQ ID NO. 46, 47, 48, 49 or an active portion thereof.

# Replacement Sheets

7. (cancelled)

8. An albumin-binding arginine deiminase fusion protein according to claim 1 wherein the linker molecule includes a sequence selected from SEQ ID NO. 50, 51, 52, 53, or serine-glycine-serine (SGS) amino acid sequence.

9. An albumin-binding arginine deiminase fusion protein according to claim 1 further comprising at least one of Poly-N or a His tag.

10. An albumin-binding arginine deiminase fusion protein according to claim 1 wherein the fusion includes a remaining portion of an intein-mediated protein ligation between the first portion and the second portion.

11. (original) An albumin-binding arginine deiminase fusion protein according to claim 10 wherein the intein-mediated protein includes a chitin binding domain.

12. An albumin-binding arginine deiminase fusion protein according to claim 1 wherein the arginine deiminase is selected from arginine deiminase produced from a *Mycoplasma*, *Lactococcus*, *Pseudomonas*, *Streptococcus*, *Escherichia*, *Mycobacterium* or *Bacillus* microorganism.

13. An albumin-binding arginine deiminase fusion protein according to claim 12 wherein the arginine deiminase is produced from *Mycoplasma arginini*, *Lactococcus lactis*, *Bacillus licheniformis*, *Bacillus cereus*, *Mycoplasma arthritidis*, *Mycoplasma hominis*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus pneumoniae*,

## Replacement Sheets

*Mycobacterium tuberculosis*, *Pseudomonas plecoglossicida*, *Pseudomonas putida*, *Pseudomonas aeruginosa* or a combination thereof.

14. (cancelled)

15. An albumin-binding arginine deiminase fusion protein according to claim 1 wherein the fusion protein is formed by reacting the arginine deiminase having a N-terminal cysteine residue with a reactive thioester at C-terminus of the albumin-binding domain so that the arginine deiminase and the albumin-binding domain are linked by a covalent bond.

16. (original) An albumin-binding arginine deiminase fusion protein according to claim 1 wherein the fusion protein is formed by reacting the albumin-binding domain having a N-terminal cysteine residue with a reactive thioester at C-terminus of the arginine deiminase so that the arginine deiminase and the albumin-binding domain are linked by a covalent bond.

17. An albumin-binding arginine deiminase fusion protein according to claim 1 wherein the fusion protein is formed by using SEQ ID NOS. 42 and 43 and by reacting the arginine deiminase having a N-terminal cysteine residue with a reactive thioester at C-terminus of the albumin-binding domain so that the arginine deiminase and the albumin-binding domain are linked by a covalent bond.

18. Use of the albumin-binding arginine deiminase fusion protein according to any one of claims 1 to 17 in preparation of a medicament for treating a cancer in a patient comprising administering a clinically effective amount of the fusion protein to the patient to reduce the availability of circulating arginine.

## Replacement Sheets

19. The use according to claim 18 wherein the cancer is pancreatic cancer, leukemia, head and neck cancer, colorectal cancer, lung cancer, breast cancer, liver cancer, nasopharyngeal cancer, esophageal cancer, prostate cancer, stomach cancer or brain cancer.

20. Use of the albumin-binding arginine deiminase fusion protein according to any one of claims 1 to 17 in preparation of a medicament for treating arginine-dependent diseases in a patient comprising administering a clinically effective amount of the fusion protein to the patient to reduce the availability of circulating arginine.

21. A method of making the albumin-binding arginine deiminase fusion protein of claim 1 comprising constructing a fusion gene coding for the albumin-binding arginine deiminase fusion protein, inserting the fusion gene into a vector, inserting the vector into a host organism and expressing a protein including the albumin-binding arginine deiminase fusion protein of claim 1.

22. The method of making the albumin-binding arginine deiminase fusion protein according to claim 21 further comprising purifying the fusion protein.

23. The method of making the albumin-binding arginine deiminase fusion protein according to claim 22 wherein the purifying is performed by chromatography.

24. The method of making the albumin-binding arginine deiminase fusion protein according to claim 22 wherein the purifying is performed from soluble fractions of crude proteins.

## Replacement Sheets

25. The method of making the albumin-binding arginine deiminase fusion protein according to claim 22 wherein the purifying is performed from insoluble fractions of crude proteins.

26. (original) A method of making the albumin-binding deiminase fusion protein of claim 1 by intein mediated protein ligation between the albumin-binding domain, albumin-binding peptide or albumin-binding protein(s) and the second portion comprising arginine deiminase.

27. A pharmaceutical composition comprising the albumin-binding arginine deiminase fusion protein of claim 1 in a pharmaceutically-acceptable carrier.

28. The pharmaceutical composition of claim 27 wherein the composition has a pH in a range of 5.5 to 9.5.

29. The pharmaceutical composition of claim 27 wherein the composition has a pH of 7.4.

30. The pharmaceutical composition of claim 27 wherein the composition has a pH of 6.5.

1/22

(A)

Native ADI

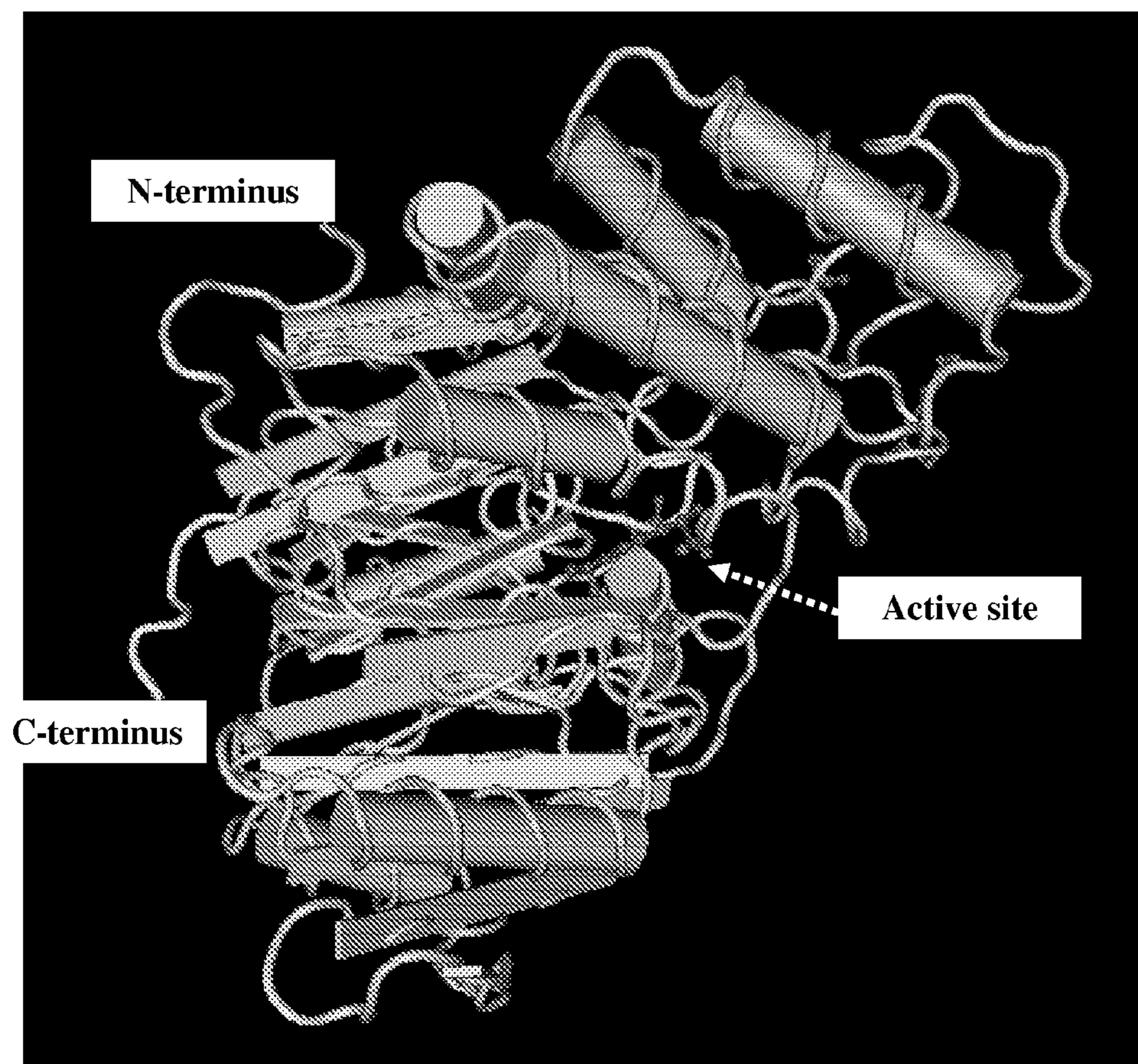
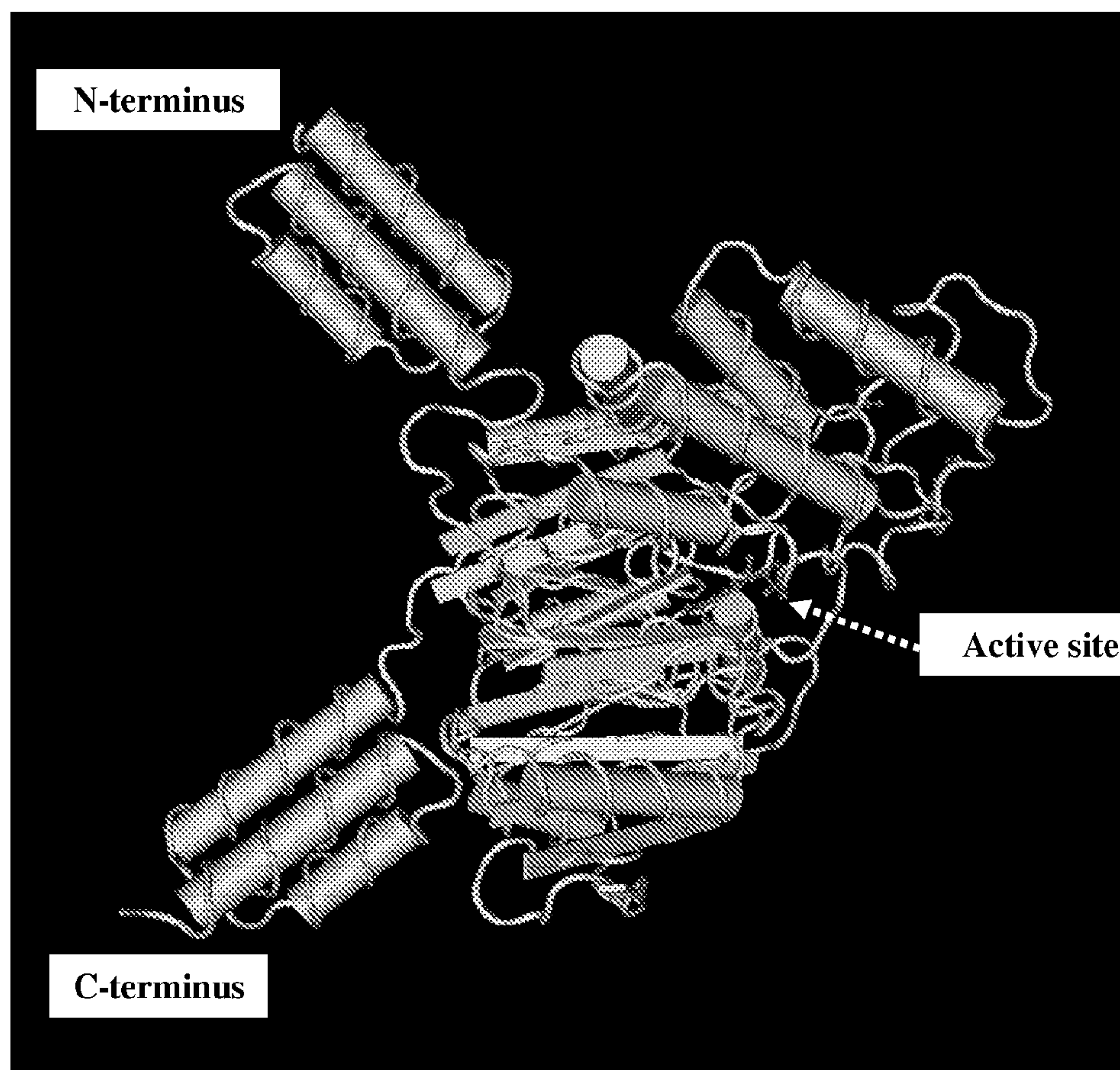


FIG. 1

2/22

(B)

AAD fusion protein with two ABD/ ABD1



SEQ ID NO: 46

ABD without linker:

LAEAKVLANRELDKYGVSDYYKNLINNAKTVEGVKALIDEILAALP

SEQ ID NO: 47

ABD with linker:

AQHDEAVDANSLAEAKVLANRELDKYGVSDYYKNLINNAKTVEGVKALIDEILAALP

SEQ ID NO: 48

ABD1 without linker:

LAEAKVLANRELDKYGVSDFYKRLINKAKTVEGVKALHILAALP

SEQ ID NO: 49

ABD1 with linker:

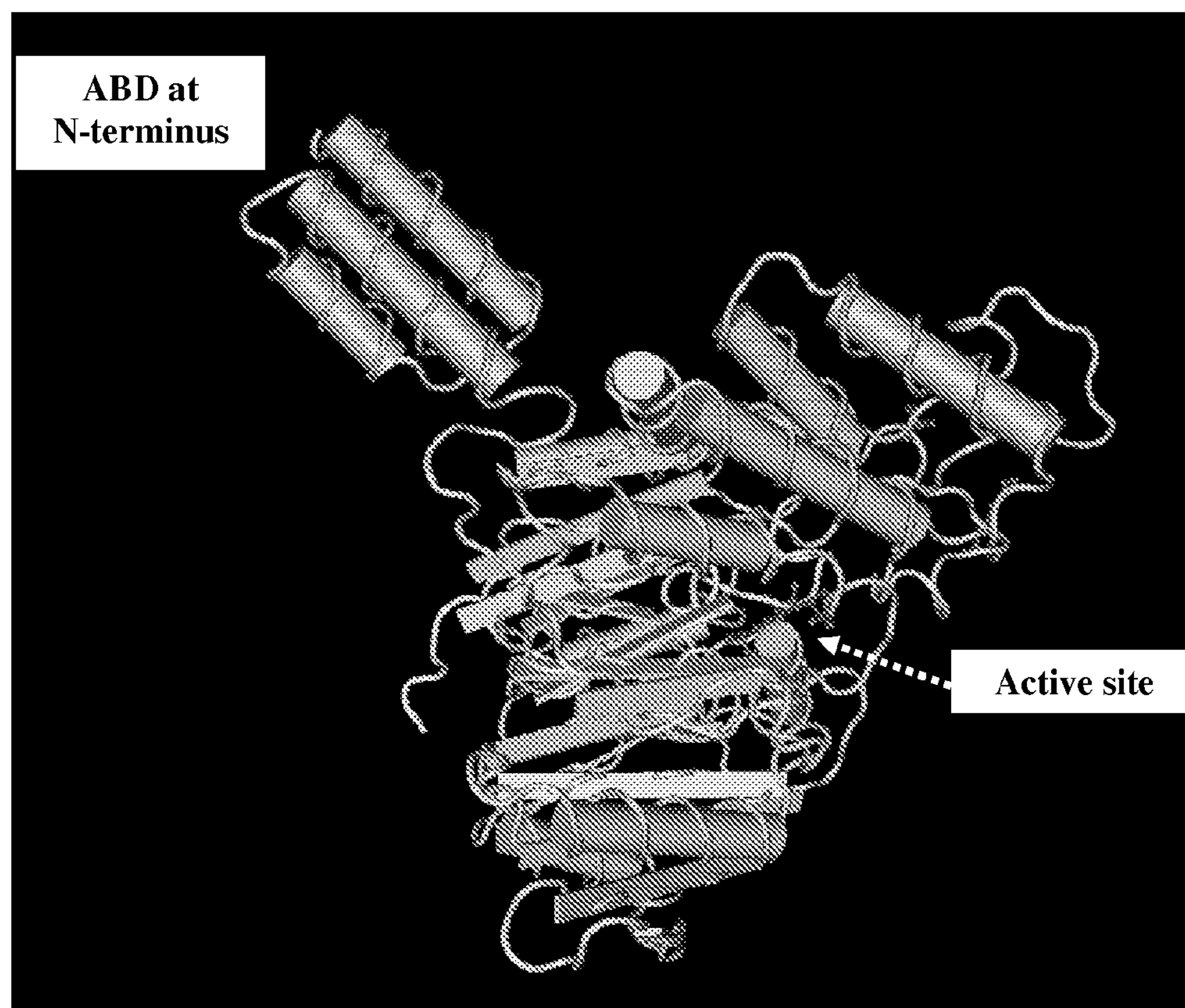
GSHHHHHHANSLAEAKVLANRELDKYGVSDFYKRLINKAKTVEGVKALHILAALP

FIG. 1 (continued)

3/22

(C)

AAD fusion protein with one ABD/ ABD1  
at N-terminus



SEQ ID NO: 46

ABD without linker:

LAEAKVLANRELDKYGVSDYYKNLINNAKTVEGVKALIDEILAALP

SEQ ID NO: 47

ABD with linker:

AQHDEAVDANSLAEAKVLANRELDKYGVSDYYKNLINNAKTVEGVKALIDEILAALP

SEQ ID NO: 48

ABD1 without linker:

LAEAKVLANRELDKYGVSDFYKRLINKAKTVEGVKALHILAALP

SEQ ID NO: 49

ABD1 with linker:

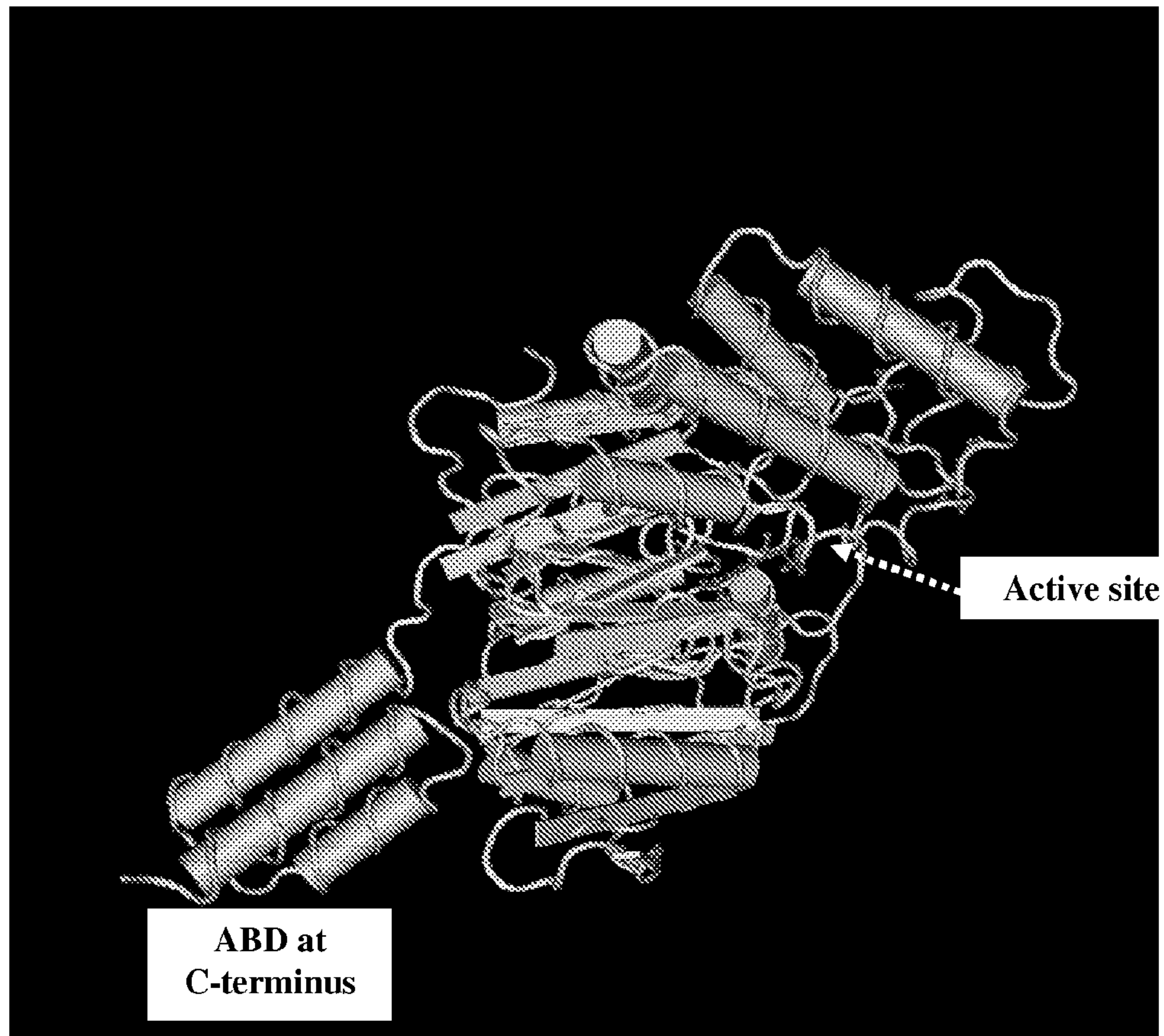
GSHHHHHHANSLAEAKVLANRELDKYGVSDFYKRLINKAKTVEGVKALHILAALP

FIG. 1 (continued)

4/22

(D)

**AAD fusion protein with one ABD/ ABD1  
at C-terminus**

**SEQ ID NO: 46****ABD without linker:** AEAQVLANRELDKYGVSDYYKNLINNAKTVEGVKALIDEILAALP**SEQ ID NO: 47****ABD with linker:**

AQHDEAVDANSLAEAQVLANRELDKYGVSDYYKNLINNAKTVEGVKALIDEILAALP

**SEQ ID NO: 48****ABD1 without linker:**

LAEAQVLANRELDKYGVSDFYKRLINKAKTVEGVKALHILAALP

**SEQ ID NO: 49****ABD1 with linker:**

GSHHHHHHANSLAEAQVLANRELDKYGVSDFYKRLINKAKTVEGVKALHILAALP

**FIG. 1 (continued)**

5/22

|                               |     |                                                                 |     |
|-------------------------------|-----|-----------------------------------------------------------------|-----|
| <i>Mycoplasma arginini</i>    | 1   | MSVTFDSKFMGIHVYSEIGELSVLVHPEGREIDYITPARLDELLFSATLESHEARKENKQ    | 60  |
| <i>Lactococcus lactis</i>     | 1   | -----MNNGINVNSEIGKIKSVLLHRRPGAVERNITPDIMKQELLEDEPYLKIAQKEHDF    | 54  |
| <i>Bacillus licheniformis</i> | 1   | -----MKHPIHVTSEIGELQIVLLKRRPGKEVENLTPDYLOQELLEDDIPYLPPIQKEHDY   | 54  |
| <i>Bacillus cereus</i>        | 1   | ----MIMTTPIHVYSEIGPLKTVMLKRRPGRELENLTPEYLERLLEDDIPFLPAVQKEHDO   | 56  |
|                               |     | *.* **** *:::*** ** *::: *** ...***. * :***.                    |     |
|                               | 61  | FVAELKANDINVVELIDVAETVYDLASQEAQDKLIEEFLEDSEPVLSSEERKVVVRNFKXA   | 120 |
|                               | 55  | FAQTERDNGAETVYIENLATEVFEKSSE-TKEEFLSHLLHEAGYRPGRTYDGL-TEYLT-    | 111 |
|                               | 55  | FAQTERNRGVEVLYLEKLAALVDK-K-LREEFVDRILKEGQADVNVVAHQTL-KEYEL-     | 110 |
|                               | 57  | FAETENQQAQAEVLYLEKLTAAALDDA-L-VREQFIDELLTESKADINGAYDRL-KEFLL-   | 112 |
|                               |     | *. *1 . :.2 : .*.1* . ::::..1* :. :. : :1*                      |     |
|                               | 121 | KWTSRELVEIMMAGITRYDL-----GIEADHELIVDEMPNLYFTIRDPFASVGMG         | 169 |
|                               | 112 | SMPTKDMVENKVIYAGVRKNELDIRTALSOMAGSDAENYFYLNPLPNAYFTIRDPQASMSGV  | 171 |
|                               | 111 | SFSNEELIQKIMGVVRKNEIETSKKTHLYE-LMEDHYPFYLDPMENLYFTIRDPAAASVGSG  | 169 |
|                               | 113 | TFDADSMVEQVMSGIRKNELEREKKSHLE-LMEDHYPFYLDPMENLYFTIRDPAAAIGSG    | 171 |
|                               |     | . :::: : .*: * :1 : . : ::::*** ***** *::: *                    |     |
|                               | 170 | VTIHYMYKVBQRETLSRFVFSNHPKLIN--TFWYBPSLKLSTEGSGEVFIYKNDTLVV      | 227 |
|                               | 172 | MTINKMTFFPARQFESLITEYVMANKPRFKD--TPIWDRNHTIRIEGGEELILNKITVAI    | 229 |
|                               | 170 | LTINKMKEPARRRRESLFMEYIINHYHFRFAKHNVPIWLSREYKFPFIEGGEELILNEETIAI | 229 |
|                               | 172 | LTINKMKEPARRRRESLFMYIINHHNPRFKGHEIFVWLDRDTKFNIEGGEELVLKEETVAI   | 231 |
|                               |     | :**.* * .*: *::: :::: *::: * : * . . ***** : : * : *:::         |     |
|                               | 228 | GVSERIDLOTVTLLAKNIVANKECEFKRIVAINVENWNTNLMHLDTWLTHLDKDKFLYSPI   | 287 |
|                               | 230 | GVSERISSKTIQNLAKELFANPLSTFDITVLAVEIENHRAMHLDITVFTMINHDQFTVFBG   | 289 |
|                               | 230 | GVSAETSAKAIERLAKNLFSSQ-NKIKKVLAIETEKRAFNMHLDITVFTMVLYENFIINHPA  | 288 |
|                               | 232 | GVSERIIAQAIERLVRNLFQRO-SRIIRVLAVEIEKSRAFNMHLDITVFTMVDRDQFTIHPA  | 290 |
|                               |     | *** ** ::: *:::.. . : ::::*** :***** :::: :** *                 |     |
|                               | 288 | ANDEVKFWDYDILVNGGAEPQP--VENGLPLEGLLQSIINKKPVLIPIAGEGASQMEIERE   | 345 |
|                               | 290 | IMDGAGNINVFILRPGQDG-EVEIEHLTDLKAALKKVLNLSELDL-IECGAGDPAAAPRE    | 347 |
|                               | 289 | IQGPKGNMNIYILEKGADEETLKITHRTSLMEALKEVLDLSELVL-IPCGGGGVIASARE    | 347 |
|                               | 291 | IQGPEGEMRIFVLERGKTADEIHITTEHNLPEVLKRTILGLSDVNL-IFCGGGGVIASARE   | 349 |
|                               |     | :.. * . * *: : . : : * ... : **                                 |     |
|                               | 346 | THFDGINKYLAIAPGVVIGYSRNEKTNAALEAAGEIKVLPFHGNQLSLGMGNARCMSEPLSE  | 405 |
|                               | 348 | QWNDGSENTLAIAPGVVITYDRNYVTVELLKEHGIKWEILSSSELGRGRGGARCM3QPLWR   | 407 |
|                               | 348 | QWNDGSENTLAIAPGVVITYDRNYVENTLLREHSGIEVIEVLSSSELGRGRGGPRCMEMPIVR | 407 |
|                               | 350 | QWNDGSENTLAIAPGVVITYDRNYISNECLREQGIKVEIIFSGELSRGRGGPRCMSEPLYR   | 409 |
|                               |     | **:* *** ** : : *.** : *. **:* . . :*. * * **** *: *            |     |
|                               | 406 | KEVKW                                                           | 410 |
|                               | 408 | EEL--                                                           | 410 |
|                               | 408 | KDI--                                                           | 410 |
|                               | 410 | EDVK-                                                           | 413 |
|                               |     | :*:                                                             |     |

FIG. 2

6/22

(A) SEQ ID NO: 36

ADI-linker-ABD 1

MSVFDSKFKGIHVYSEIGELESVLVHEPGREIDYITPARLDELLESAILES HDARKEHKQ  
 FVAELKANDINVVELIDLVAETDYLASQEAKDKLIEEFLEDSEPVLS EENKVVVRNFLKA  
 KKT SRELVEIMMAGITKYDLGIEADHELIVDPMPNLYFTRDPFASVGNGVTIHYMRYKVR  
 QRETLESRFVFSNHPKLINTPWYYDPSLKLSIEGGDVFIYNNDTLVVGVSERTDLQTVTL  
 LAKNIVANKECEFKRIVAINVPKWTNLMHLDTWLTMLDKDKFLYSP IANDVFKFWDYDLV  
 NGGAEPQPVENGLPLEGLLQSIINKKPVLIPIAGEGASQMEIERETHFDGTNYLAIRPGV  
 VIGYSRNEKTNAALEAAGIKVLPFHGNQLSLGMGNARCM SMPLSRKDVKW GSHHHHHHHAN  
SLAEAKVLANRELDKYGVSDFYKRLINKAKTVEGVEALKLHILAALF

(ABD 1: high affinity albumin binding domain; the linker is underlined.)

Linker 1 (SEQ ID NO: 50): GSHHHHHHANS

(B) SEQ ID NO: 37

ADI-linker-ABD

MSVFDSKFKGIHVYSEIGELESVLVHEPGREIDYITPARLDELLESAILES HDARKEHKQ  
 FVAELKANDINVVELIDLVAETDYLASQEAKDKLIEEFLEDSEPVLS EENKVVVRNFLKA  
 KKT SRELVEIMMAGITKYDLGIEADHELIVDPMPNLYFTRDPFASVGNGVTIHYMRYKVR  
 QRETLESRFVFSNHPKLINTPWYYDPSLKLSIEGGDVFIYNNDTLVVGVSERTDLQTVTL  
 LAKNIVANKECEFKRIVAINVPKWTNLMHLDTWLTMLDKDKFLYSP IANDVFKFWDYDLV  
 NGGAEPQPVENGLPLEGLLQSIINKKPVLIPIAGEGASQMEIERETHFDGTNYLAIRPGV  
 VIGYSRNEKTNAALEAAGIKVLPFHGNQLSLGMGNARCM SMPLSPKDVKWA AQHDEAVDAN  
SLAEAKVLANRELDKYGVSDYYRNLIINAKTVEGVKALIDEILAALF

(ABD: albumin binding domain; the linker is underlined.)

Linker 2 (SEQ ID NO: 51): AQHDEAVDANS

FIG. 3

7/22

(C) SEQ ID NO: 38



MSVFDSKFKGIHVYSEIGELESVLVHEPGREIDYITPARLDELLFSAILSHDARKEHKQ  
 FVAELKANDINVVELIDLVAETYDLASQEAKDKLIEEFLEDSEFVLSEEHKVVVRNFLKA  
 KKTSRELVEIMMAGITKYDLGTEADHELIVDFMPNLYFTRDPFASVGNGVTIHYMRYKVR  
 QRETLFSRFVFSNHPKLINTPWYYDPSLKLSIEGGDVFIYNNDTLVVGVSERTDLQTVTL  
 LAKNIVANKECEFKRIVAINVPKWTNLMHLDTWLTMLDKDKFLYSPIANDVFKFWDYDLV  
 NGGAEPQPVENGLPLEGLLQSIINKKPVLIPIAGEGASQMEIERETHFDGTNYLAIRPGV  
 VIGYSPNEKTNAALEAAGIKVLPFHGNQLSLGMGNARCMSPLSRKDVKWAQHDEAVDAN  
SLAEAKVLANRELDKYGVSDYYKNLINNAKTVEGVKALIDEILAALP

(ABD: albumin binding domain; the linker is underlined.)

(D) SEQ ID NO: 39



MSVFDSKFKGIHVYSEIGELESVLVHEPGREIDYITPARLDELLFSAILSHDARKEHKQ  
 FVAELKANDINVVELIDLVAETYDLASQEAKDKLIEEFLEDSEFVLSEEHKVVVRNFLKA  
 KKTSRELVEIMMAGITKYDLGTEADHELIVDFMPNLYFTRDPFASVGNGVTIHYMRYKVR  
 QRETLFSRFVFSNHPKLINTPWYYDPSLKLSIEGGDVFIYNNDTLVVGVSERTDLQTVTL  
 LAKNIVANKECEFKRIVAINVPKWTNLMHLDTWLTMLDKDKFLYSPIANDVFKFWDYDLV  
 NGGAEPQPVENGLPLEGLLQSIINKKPVLIPIAGEGASQMEIERETHFDGTNYLAIRPGV  
 VIGYSPNEKTNAALEAAGIKVLPFHGNQLSLGMGNARCMSPLSRKDVKWAQHDEAVDAN  
SLAEAKVLANRELDKYGVSDYYKNLINNAKTVEGVKALIDEILAALP

(ABD: albumin binding domain; the linker is underlined.)

FIG. 3 (continued)

8/22

(E) SEQ ID NO: 40



MTTHHHHHDEAVDANSIAEAKVLANRELDKYGVSDYYKNLINNAKTVEGVKALIDEILAAAL  
PSGSMNNNNNGSGGSVFD SKFKGIHVYSEIGELESVLVHEPGREIDYITPARLDELCSA  
I LESH DARKEHKQFVAELKANDINVVELIDLVAETYDLASQEAKDKLIEEFLEDSEPVLS  
EEHKVVVRNFLKAKKTSRELVEIMMAGITKYDLGIEADHELIVDPMPNLYFTRDPFASVG  
NGVTIHYMRYKVRQRETLFSRFVFSNHPKLINTPWYYDPSLKLSIAGGDVFIYNNDTLVV  
GVSERTDLQTVTLLAKNIVANKESSEFKRIVAINVPKWTNLMHLDTWLTMLDKDKFLYSPI  
ANDVFKFWDYDLVNGGAEPQPVENGLPLEGLLQSIINKKPVLIP IAGEGASQMEIERETH  
FDGTNYLAIRPGVVIGYSRNEKTNAALEAAGIKVLPFHGNQLSLGMGNARCMSPLSRKD  
VKW

(ABD: albumin binding domain; the linker between His and ABD is underlined with solid line while the linker between Poly-N and ADI is underlined with dotted line.)

Linker 3 (SEQ ID NO: 52): DEAVDANS; Linker 4: SGS; Linker 5 (SEQ ID NO: 53): GSGG

**FIG. 3 (continued)**

9/22

(F) SEQ ID NO: 41



MG DEAVDANS LAEAKVLANRELDKYGVSDYYKNLINNAKTVEGVKALIDEILAALP  
 SG SGG KHP IHVTSEIGELQTVLLKRPGKEVENLTPDYLQQLLFDDIPYLP I IQK  
 EHDYFAQTLRNRGVEVLYLEKLAAEALVDKKLREEFVDRI LKEGQADVNVAHQTLKEYLLSF  
 SNEELIQKIMETGGVRKNEIETSKKTHLYELMETEDHYPFYLDPMETPNLYFTRDPAASVGD  
 GLTINKMETREPARRRESLFMETEYI IKYHPRFEKHNVP IWLDRDYKFPIEGGDELILNEET  
 IAIGVSARTSAKAIERLAKNLF SRQNKIKKVLAIE IPKCRAFMETHLDTVFTMETVDYDKFT  
 IHPAIQGPKGNMETNIYILEKGSDEETLKITHRTSLMETEALKEVLGLSELVLIPCGGGDVI  
 ASAREQWNDGSNTLAIAPGVVVTYDRNYVSNTLLREHGIEVIEVLSSELSRGRGGPRCMETS  
 METPIVRKDI

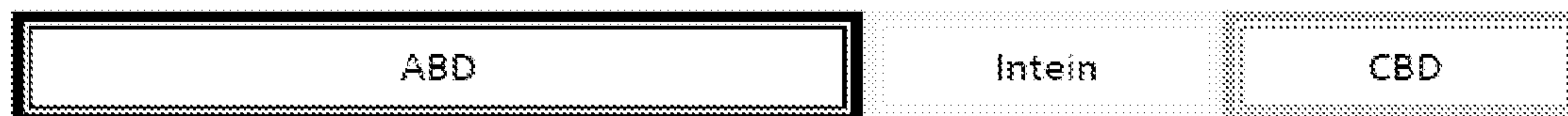
(ABD: *avoumin* binding domain; the linker between His and ABD is underlined with solid line  
 while the linker between Poly-N and bcADI is underlined with dotted line.)

Linker 3 (SEQ ID NO: 52): DEAVDANS; Linker 4: SGS; Linker 5 (SEQ ID NO: 53): GSGG

**FIG. 3 (continued)**

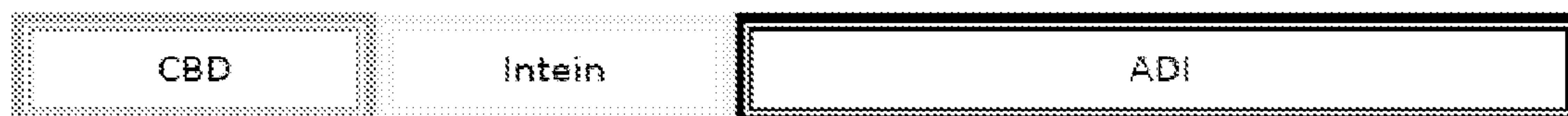
10/22

(A) SEQ ID NO: 42



MAQHDEAVDANSLAEAKVLANRELDKYGVSDYYKNLINNAKTVEGVKALIDEILAAALPEF  
 LEGSSCITGDALVALPEGESVRIADIVPGARPNSDNAIDLKVLDRHGNPVLADRLPHSGE  
 HPVYTVRTVEGLRVTGTANHPLLCLVDVAGVPTLLWKLIDEIKPGDYAVIQRSAFSVDCA  
 GFARGCKPEFAPTTYTVGVPGGLVRFLEAHRDPDAQAIADELTDGRFYAKVASVTDAGVQ  
 PVYSLRVDTADHAFITNGFVSHATCLTGLNSGLTNPQVSAWQVNTAYTAGQLVTYNGKT  
 YKCLQPHTSLAGWEEPSNVEALWQLQGEFITTITK

(B) SEQ ID NO: 43

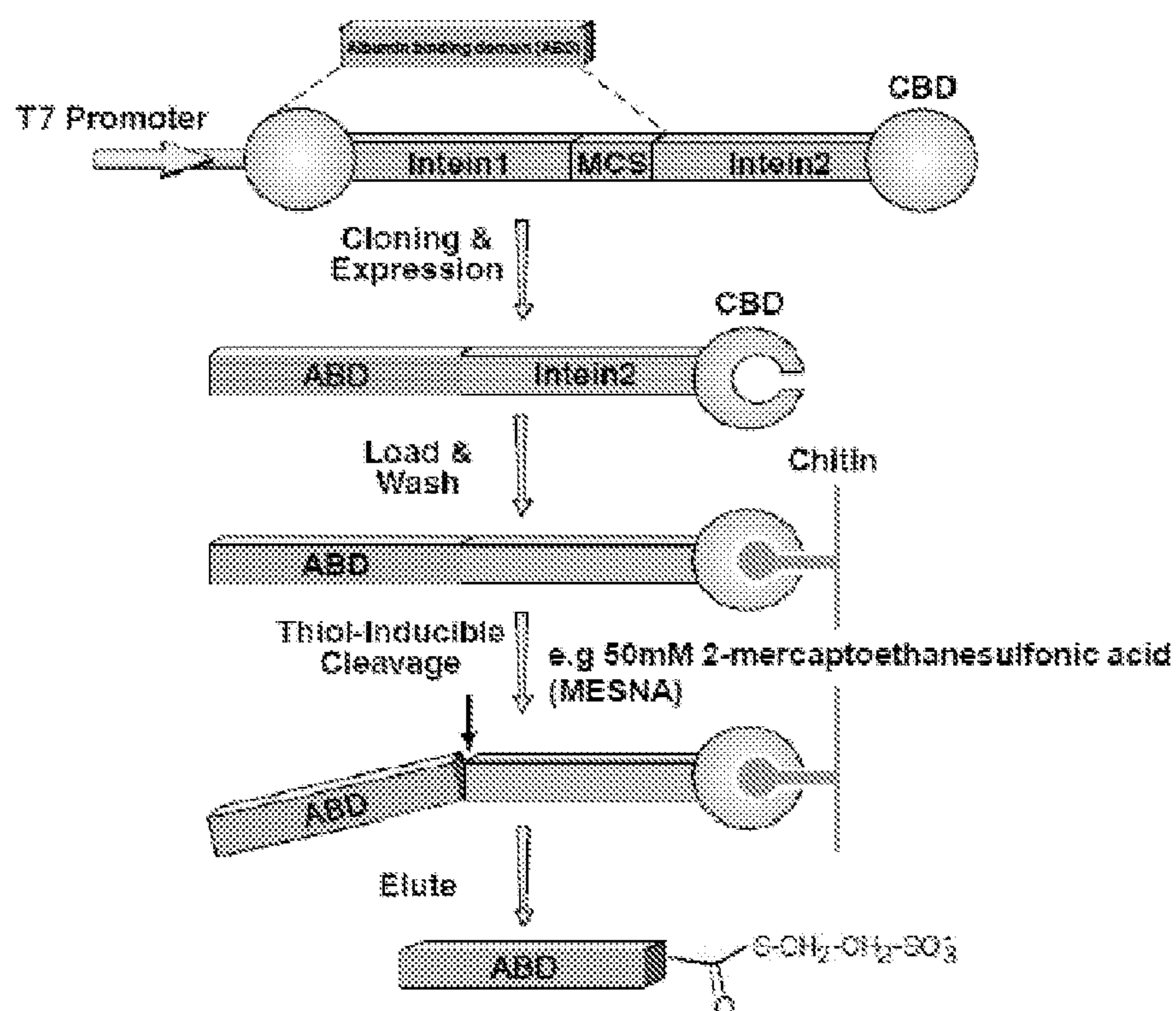


MEIEEGKLTNPQVSAWQVNTAYTAGQLVTYNGKTYKCLQPHTSLAGWEEPSNVEALWQLON  
 NQNGLELPESGAISGDSLSLASTGKRVSINKDLLDENDFEIWAINEQTMKLESKVERV  
 FCTGKKLVYILKTRLGRTIKATANHRFLTIDGWRLDELSLKEHIALPRKLESSSLQLSP  
 EIEKLSQSDIYWDSIVSITETGVVEEVEDLTVPGPHNEVANDIIVHNCSVFDSKFKGIHVY  
 SEIGELESVLVHEPGREIDYITPARLDELQFSAILSHDARKEHKQFVAELKANDINVVE  
 LIDLVAETYDLASQEAKDKLIEEFLEDSEPVLSSEHKVVVRNFKAKKTSPELVEIMMAG  
 ITKYDLGIEADHELIVDFMPNLYFTRDPFASVGNVGTIHYMPYKVRQRETLSRFVFSNH  
 PKLINTFWYYDPSLKLSIAGGDVFIYNNDTLVVGVSEPTDLQTVTLLAKNIVANKESSEFK  
 RIVAINVPKWTNLMHLDTWLTMLDKDKELYSPANDVFKFWDYDLVNGGAEPQPVENGLE  
 LEGLLQSIINKKPVLIPIAGEGASQMEIERETHFDGTNYLAIRPGVVIGYSRNEKTNAAL  
 EAAGIKVLFFHGNQLSLGMGNARCMSPMLSEKDVKW

FIG. 4

11/22

(C)

C-Terminal  
fusion

(D)

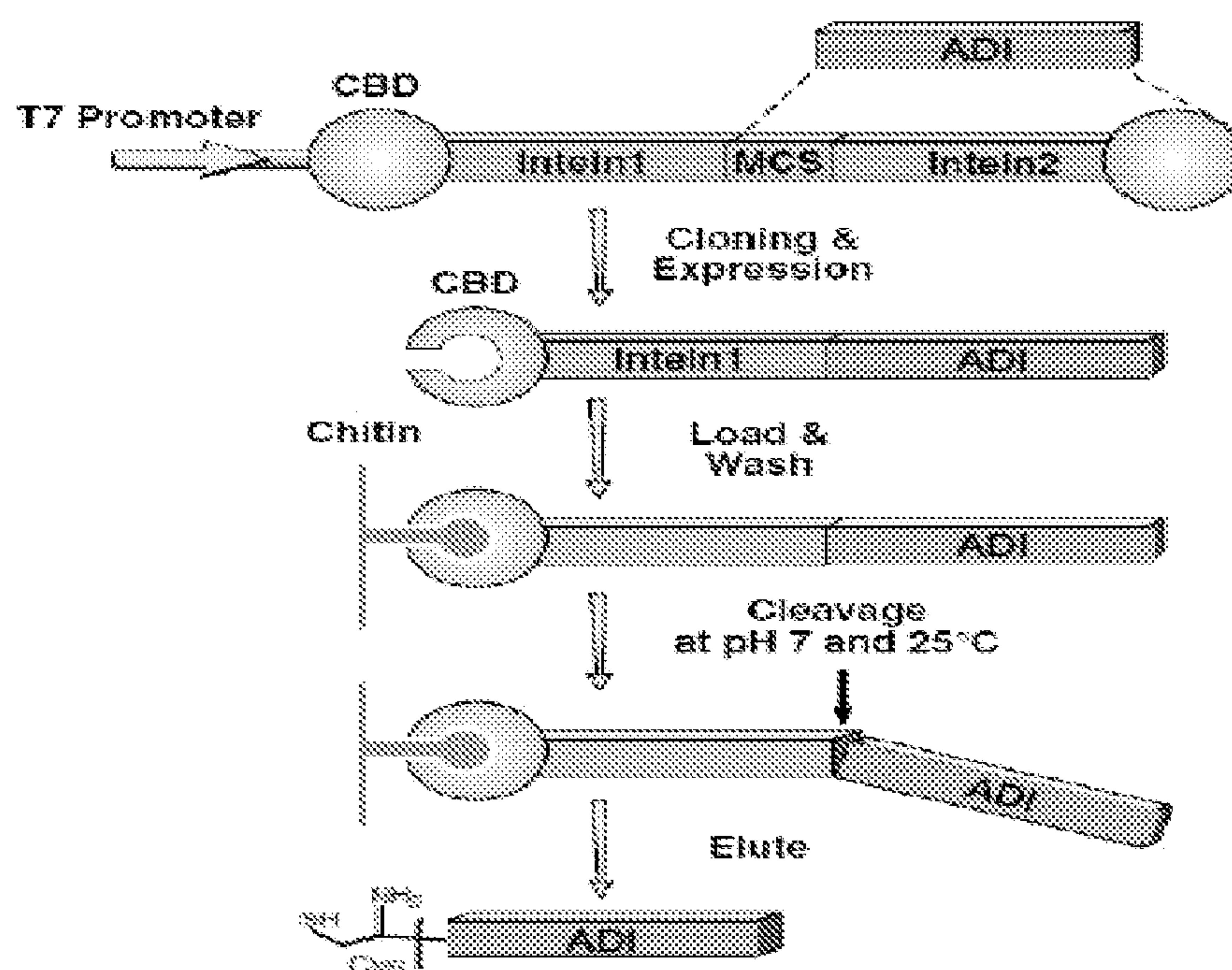
N-Terminal  
fusion

FIG. 4 (continued)

12/22

(E)

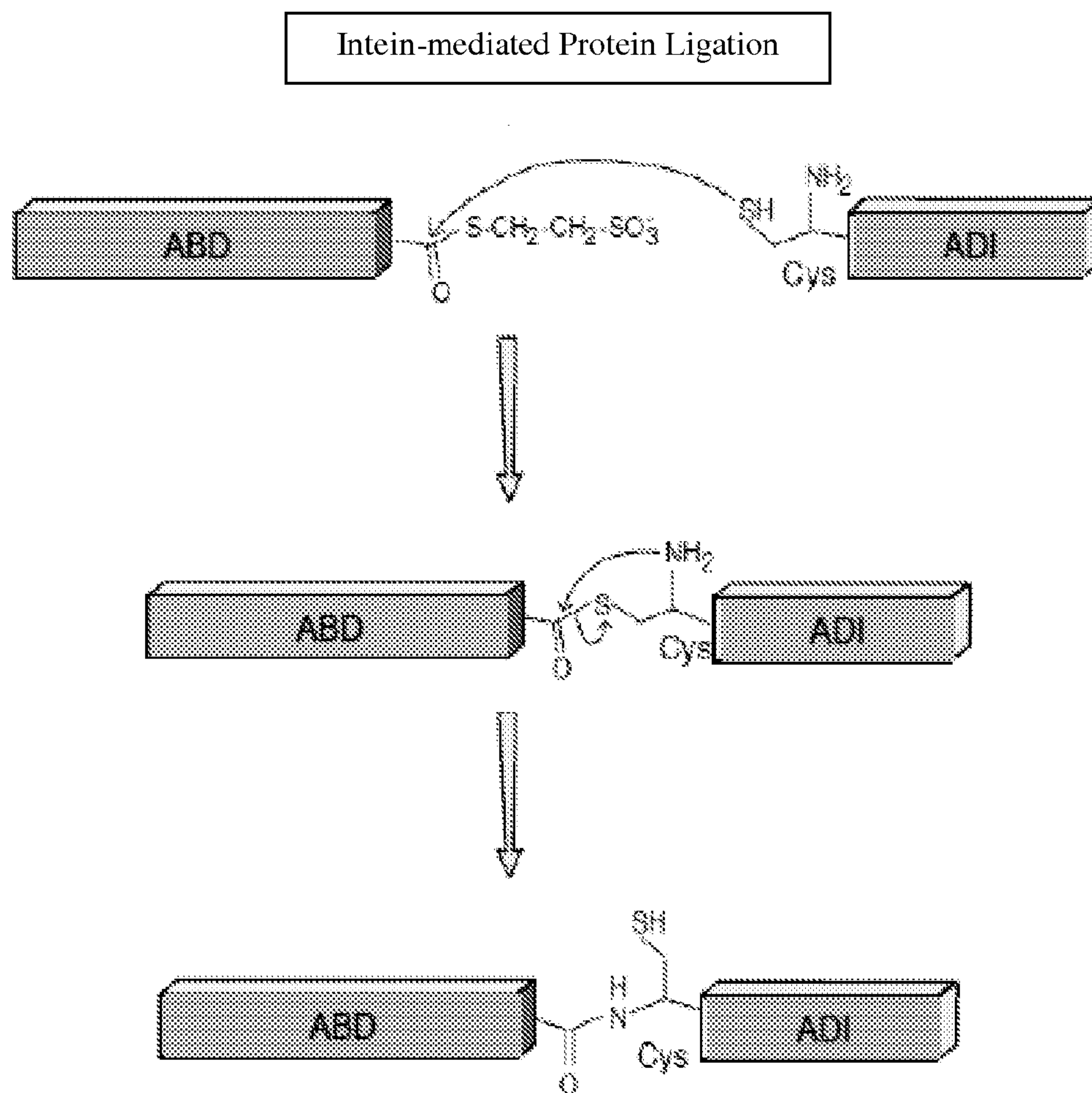


FIG. 4 (continued)

13/22

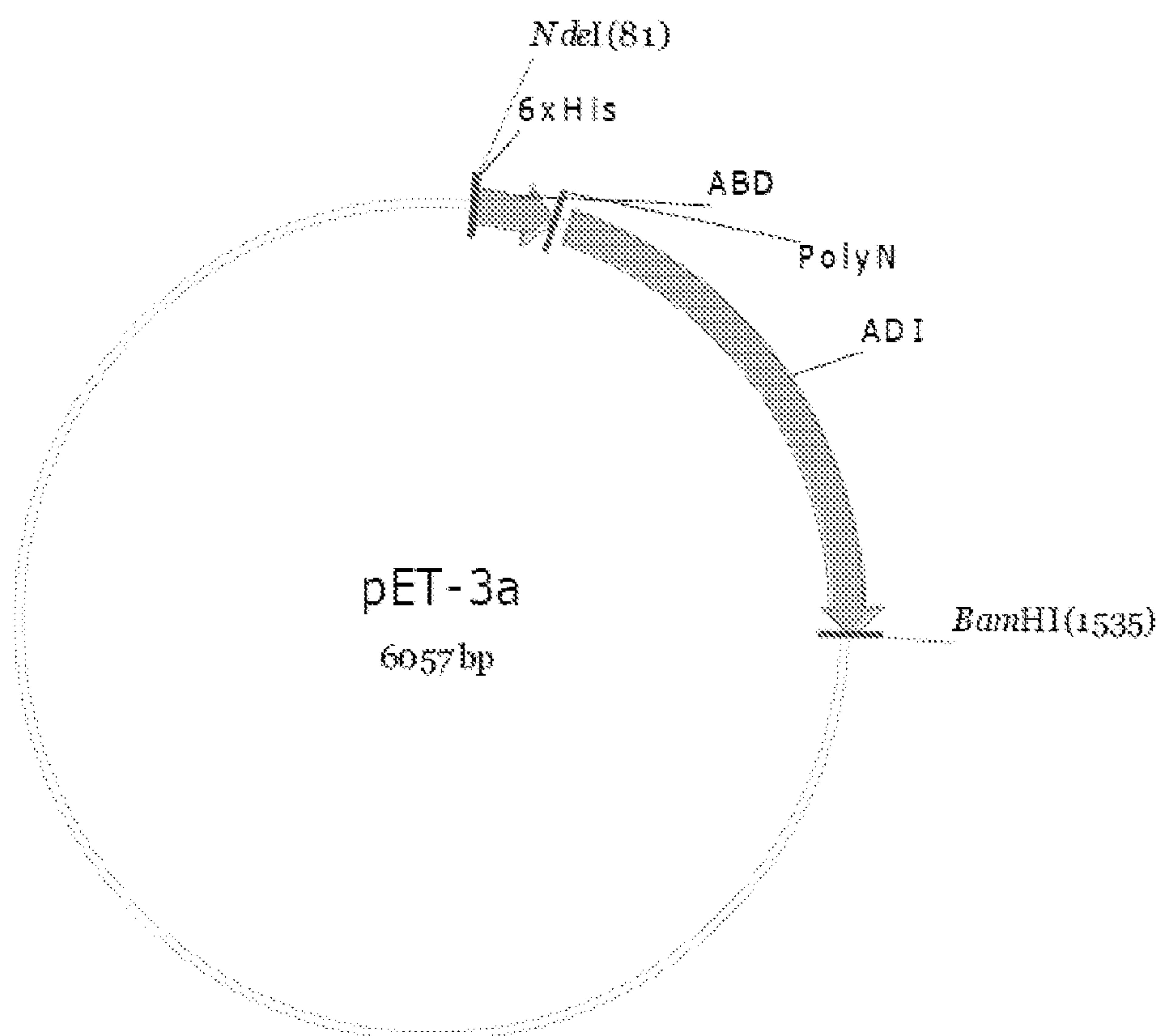
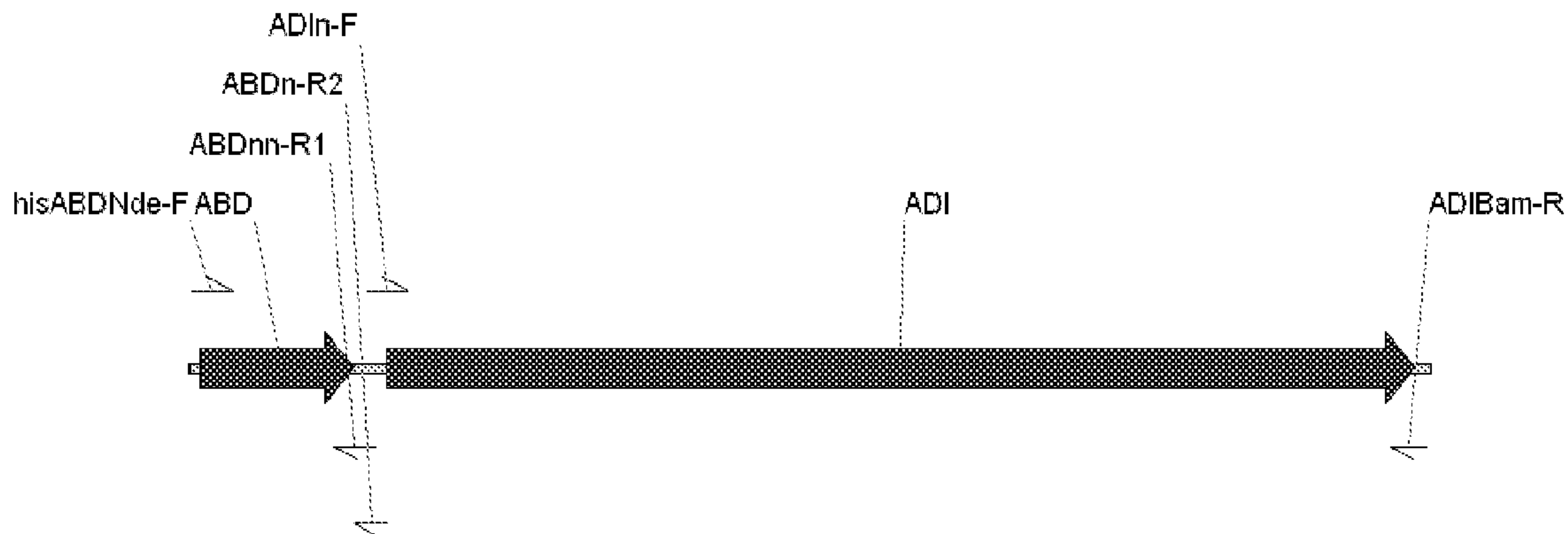


FIG. 5

14/22

## (A) GENE MAP

(B) Nucleotide sequence of **His-ABD-PolyN-ADI** (1484 bp):

(SEQ ID NO: 44)

5' -

ATGCATCATCACCATCACCATGATGAAGCCGTGGATGCGAATTCCTTAGCTGAAGCTAAAGTCT  
 TAGCTAACAGAGAACTTGACAAATATGGAGTAAGTGACTATTACAAGAACCTAATCAACAATGC  
 CAAAACTGTTGAAGGTGTAAAAGCACTGATAGATGAAATTTTAGCTGCATTACCTTCGGGTAGT  
 AACAACAATAATAACAATGGTAGCGGCGGTTCTGTATTTGACAGTAAATTTAAAGGAATTCACG  
 TTTATTCAGAAATTGGTGAATTAGAATCAGTTCTAGTTCACGAACCAGGACGCGAAATTGACTA  
 TATTACACCAGCTAGACTAGATGAATTATGCTTCTCAGCTATCTTAGAAAGCCACGATGCTAGA  
 AAAGAACACAAACAATTCGTAGCAGAATTAAAAGCAAACGACATCAATGTTGTTGAATTAATTG  
 ATTTAGTTGCTGAAACATATGATTTAGCATCACAAGAAGCTAAAGACAAATTAATCGAAGAATT  
 TTTAGAAGACTCAGAACCAGTTCTATCAGAAGAACACAAAGTAGTTGTAAGAACTTCTTAAAA  
 GCTAAAAAACATCAAGAGAATTAGTAGAAATCATGATGGCAGGGATCACAAAATACGATTTAG  
 GTATCGAAGCAGATCACGAATTAATCGTTGACCCAATGCCAAACCTATACTTCACACGTGACCC  
 ATTTGCATCAGTAGGTAATGGTGTAAACAATCCACTACATGCGTTACAAAGTTAGACAACGTGAA  
 ACATTATTCTCAAGATTTGTATTCTCAAATCACCCATAACTAATTAACACTCCATGGTACTACG  
 ACCCTTCACTAAAATTATCAATCGCAGGTGGGGACGTATTTATCTACAACAATGACACATTAGT  
 AGTTGGTGTCTTCTGAAAGAACTGACTTACAAACAGTTACTTTATTAGCTAAAAACATTGTTGCT  
 AATAAAGAATCTGAATTCAAACGTATTGTTGCAATTAACGTTCCAAAATGGACAACTTAATGC  
 ACTTAGACACATGGCTAACAATGTTAGACAAGGACAAATTCCTATACTCACCAATCGCTAATGA  
 CGTATTTTAAATTCTGGGATTATGACTTAGTAAACGGTGGAGCAGAACCACAACCAGTTGAAAAC  
 GGATTACCTCTAGAAGGATTATTACAATCAATCATTAACAAAAAACAGTTTTAATTCCTATCG  
 CAGGTGAAGGTGCTTCACAAATGGAAATCGAAAGAGAAACACACTTCGATGGTACAACTACTT  
 AGCAATTAGACCAGGTGTTGTAATTGGTTACTCACGTAACGAAAAACAAACGCTGCTCTAGAA  
 GCTGCAGGCATTAAAGTTCTTCCATTCCACGGTAACCAATTATCATTAGGTATGGGTAAACGCTC  
 GTTGTATGTCAATGCCTTTATCACGTAAAGATGTTAAGTGGTAA-3'

FIG. 6

15/22

(C) Amino acid sequence of **His-ABD-PolyN-ADI**:

(SEQ ID NO: 40)

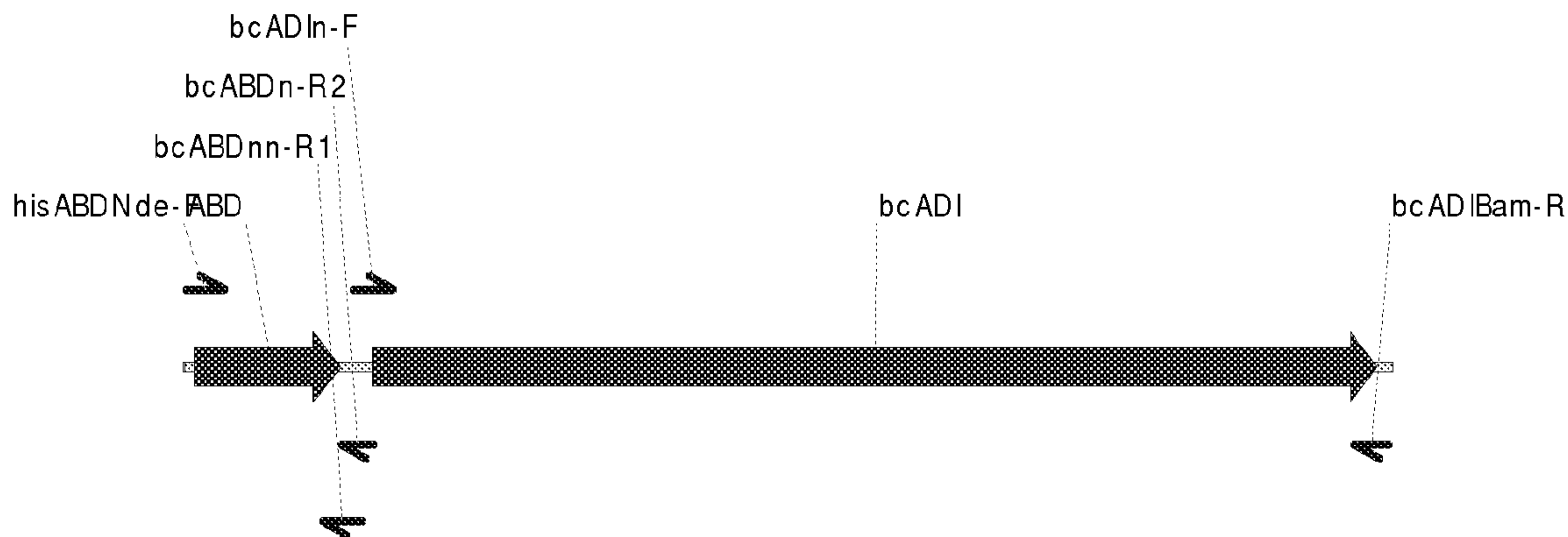
MHHHHHDEAVDANSLAEAKVLANRELDKYGVSDYYKNLINNAKTVEGVKALIDEILAALPSGSNNNNNNGSGGSVF  
DSKFKGIHVYSEIGELESVLVHEPGREIDYITPARLDELCFSAILESHDARKEHKQFVAELKANDINVVELIDLVAET  
YDLASQEAKDKLIEEFLEDSEPVLSEEHKVVRNFLKAKKTSRELVEIMMAGITKYDLGIEADHELIVDPMPNLYFTR  
DPFASVGNGVTIHYMRYKVRQRETLSRFVFSNHPKLINTPWYYDPSLKLSIAGGDVFIYNNDTLVVGVSERTDLQTV  
TLLAKNIVANKESSEFKRIVAINVPKWTNLMHLDTWLTMLDKDKFLYSPIANDVFKFWDYDLVNGGAEPQPVENGLPLE  
GLLQSIINKKPVLIPAGEGASQMEIERETHFDGTNYLAIRPGVVIGYSRNEKTNAALEAAGIKVLPFHGNQLSLGMG  
NARCMSMPLSRKDVKW

(PolyN with linker: SGSNNNNNNGSGG)

**FIG. 6 (continued)**

16/22

## (A) GENE MAP

(B) The nucleotide sequence of **His-ABD-PolyN-bcADI (1512 bp)**:

(SEQ ID NO: 45)

5' –  
 ATGGGTCATCATCACCATCACCATGATGAAGCCGTGGATGCGAACAGCTTAGCTGAAGCTAAAG  
 TCTTAGCTAACAGAGAACTTGACAAATATGGAGTAAGTGACTATTACAAGAACCTAATCAACAA  
 TGCCAAAACIGTTGAAGGTGTAAAAGCACTGATAGATGAAATTTTAGCTGCATTACCTTCGGGT  
 AGTAACAACAATAATAACAATGGTAGCGGCGGTAAACATCCGATACATGTTACTTCAGAAATTG  
 GGGAATTACAAACGGTTTTTATTAAAACGACCGGGTAAAGAAGTGGAAGAACTTGACGCCAGATTA  
 TTTGCAGCAATTATTATTTGACGATATTCCATACCTACCAATTATTCAAAAAGAGCATGATTAT  
 TTTGCACAAACGTTACGCAATCGGGGTGTTGAAGTTCTTTATTTAGAAAACTAGCCGCTGAGG  
 CGTTAGTAGATAAAAAACTTCGAGAAGAATTTGTTGATCGTATTTTAAAAGAAGGACAGGCCGA  
 CGTAAATGTTGCACATCAAACTTTAAAAGAATATTTACTTTTCTTTTCAAATGAAGAATTAATT  
 CAAAAAATTATGGGCGGTGTACGGAAAAACGAAATTGAAACAAGTAAGAAGACACATTTATATG  
 AATTAATGGAAGATCATTATCCGTTTTACTTAGATCCAATGCCTAATTTATATTTTACTCGTGA  
 TCCAGCAGCTAGCGTGGGCGATGGCTTAACGATAAATAAGATGAGAGAACCAGCGCGTAGACGT  
 GAATCATTATTCATGGAGTACATCATTAAATATCATCCAAGATTTGAAAAACATAATGTACCAA  
 TCTGGTTAGATCGTGATTATAAATTTCCAATTGAAGGTGGCGACGAGCTAATTTTAAATGAAGA  
 AACAATTGCGATTGGAGTATCTGCTCGTACTTCAGCTAAAGCAATTGAACGTTTAGCAAAAAAT  
 CTCTTTAGCCGACAAAATAAAATTAAGAAAGTGTTAGCAATAGAAATTCCAAAATGCCGAGCAT  
 TTATGCATTTAGATACAGTATTTACAATGGTTGATTATGATAAGTTTACAATTCACCCAGCTAT  
 TCAAGGGCCAAAAGGGAATATGAATATTTATATTTTAGAAAAAGGATCAGATGAGGAAACTCTT  
 AAAATTACACATCGTACTTCTTTAATGGAAGCATTAAAAGAGGTATTAGGCTTAAGTGAATTAG  
 TTCTTATTCCATGTGGAGGAGGAGATGTAATTGCTTCTGCTCGTGAACAATGGAATGATGGCTC  
 GAACACATTAGCAATCGCGCCAGGTGTAGTTGTTACATATGATCGCAACTATGTATCCAATACG  
 TTATTACGGGAACACGGTATAGAAGTGATTGAGGTGCTAAGTTCAGAATTATCTCGTGGTCGTG  
 GGGGTCCACGTTGCATGAGTATGCCAATTGTTTCGTAAAGATATTTAA-3'

FIG. 7

17/22

(C) The amino acid sequence of **His-ABD-PolyN-bcADI**:

(SEQ ID NO: 41)

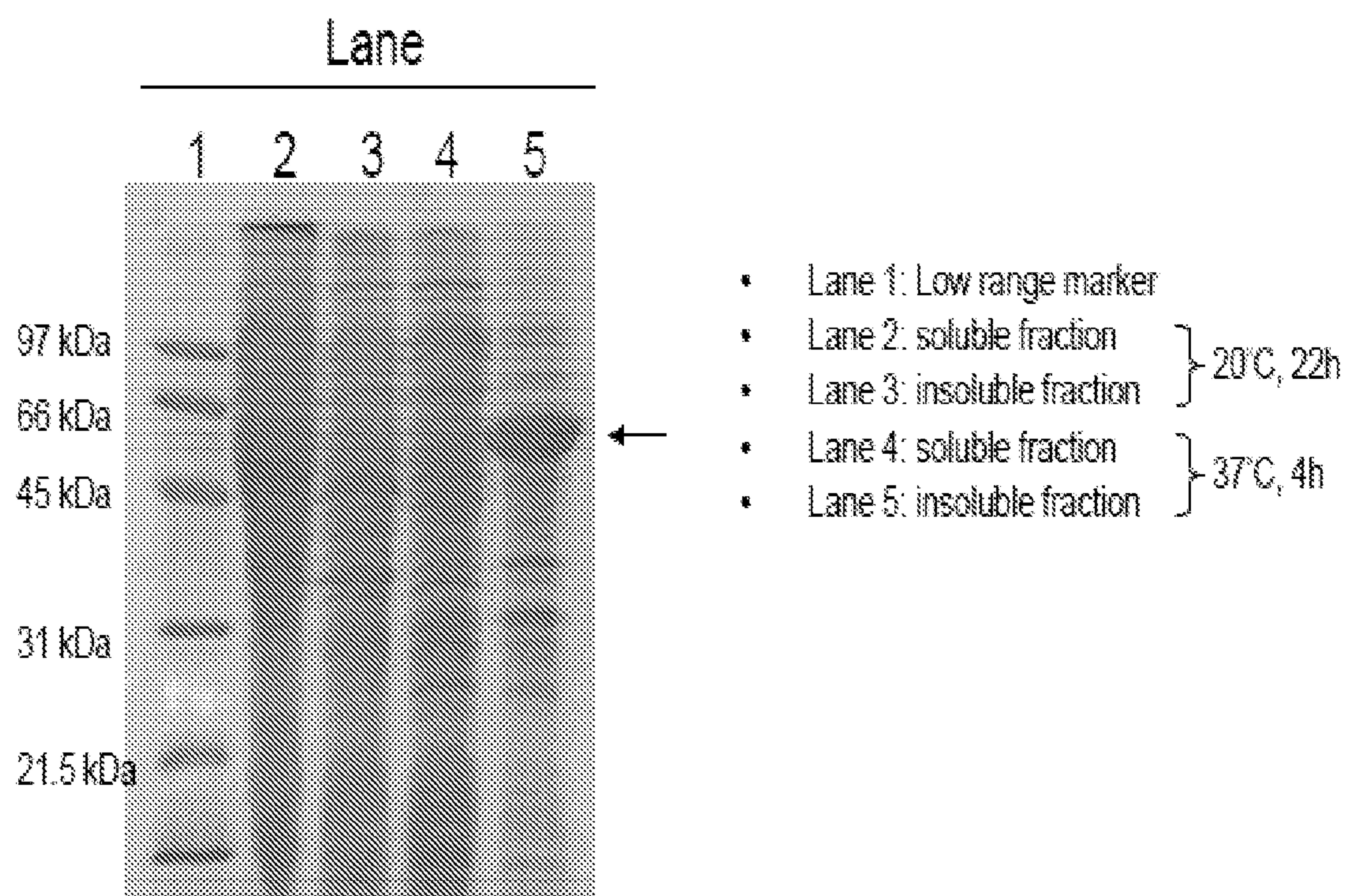
MGHHHHHHDEAVDANSLAEAKVLANRELDKYGVSDYYKNLINNAKTVEGVKALIDEILAALPSGSNNNNNNGSGGKH  
PIHVTSEIGELQTVLLKRPGKEVENLTPDYLQQLFDDIPYLPITQKEHDYFAQTLNRNGVEVLYLEKLAAEALVDKK  
LREEFVDRILKEGQADVNVAHQTLKEYLLSFSNEELIQKIMETGGVRKNEIETSKKTHLYELMETEDHYPFYLDPMET  
PNLYFTRDPAASVGDLTINKMETREPARRRESLFMETEYIIKYHPRFEKHNVPWLD RDYKFP IEGGDELILNEETI  
AIGVSARTSAKAIERLAKNLF SRQNKIKKVL AIEIPKCR AFMETHLDTVFTMETVDYDKFTIHPAIQGPKGNMETNIY  
ILEKGSDEETLKITHRTSLMETEALKEVLGLSELVLPCGGGDVIASAREQWNDGSNTLAIAPGVVVV TYDRNYVSNTL  
LREHGIEVIEVLSSSELSRGRGGPRCMETSMETPIVRKDI

(PolyN with linker: SGSNNNNNNGSGG)

**FIG. 7 (continued)**

18/22

(A)



(B)

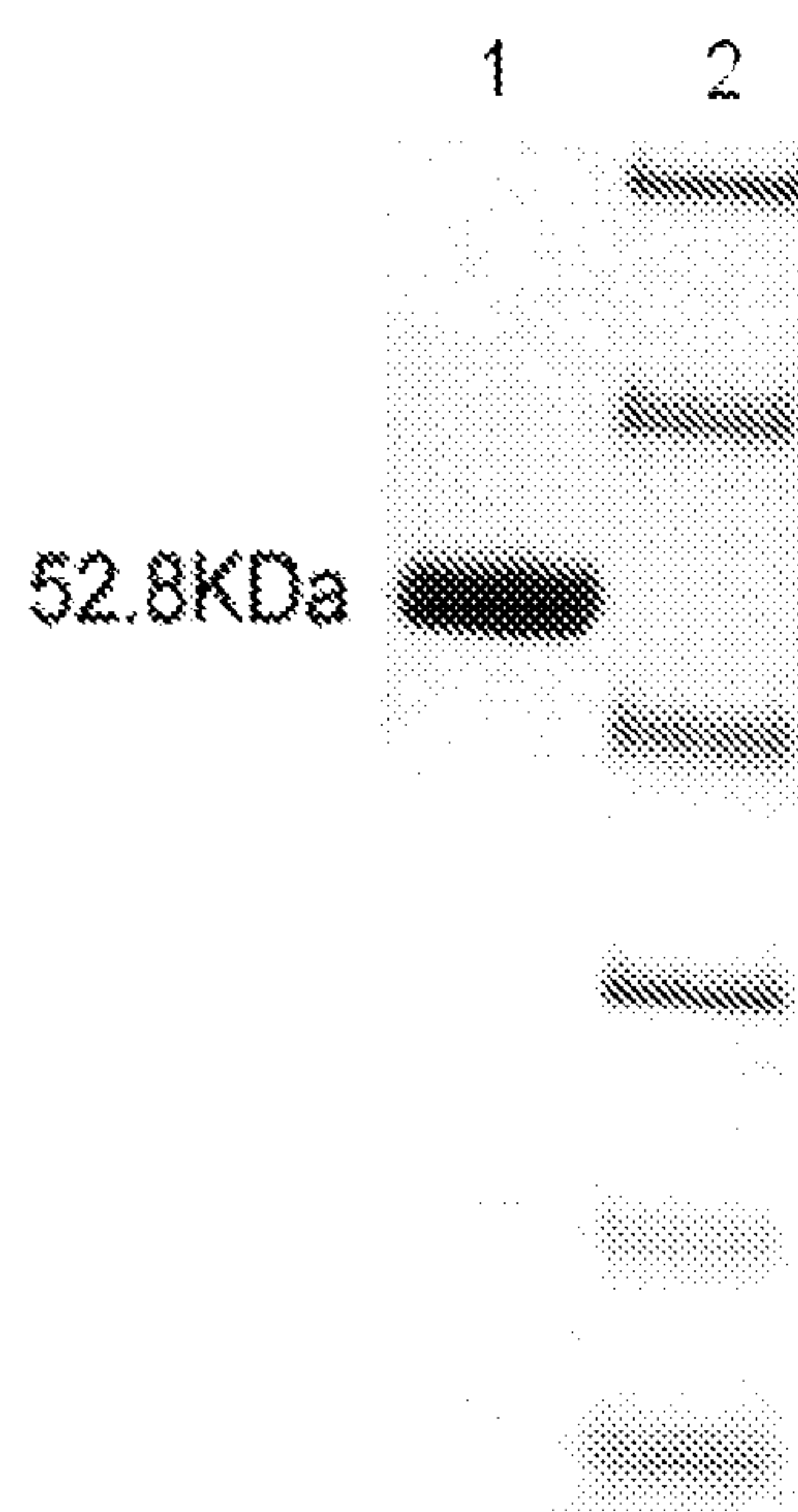


FIG. 8

19/22

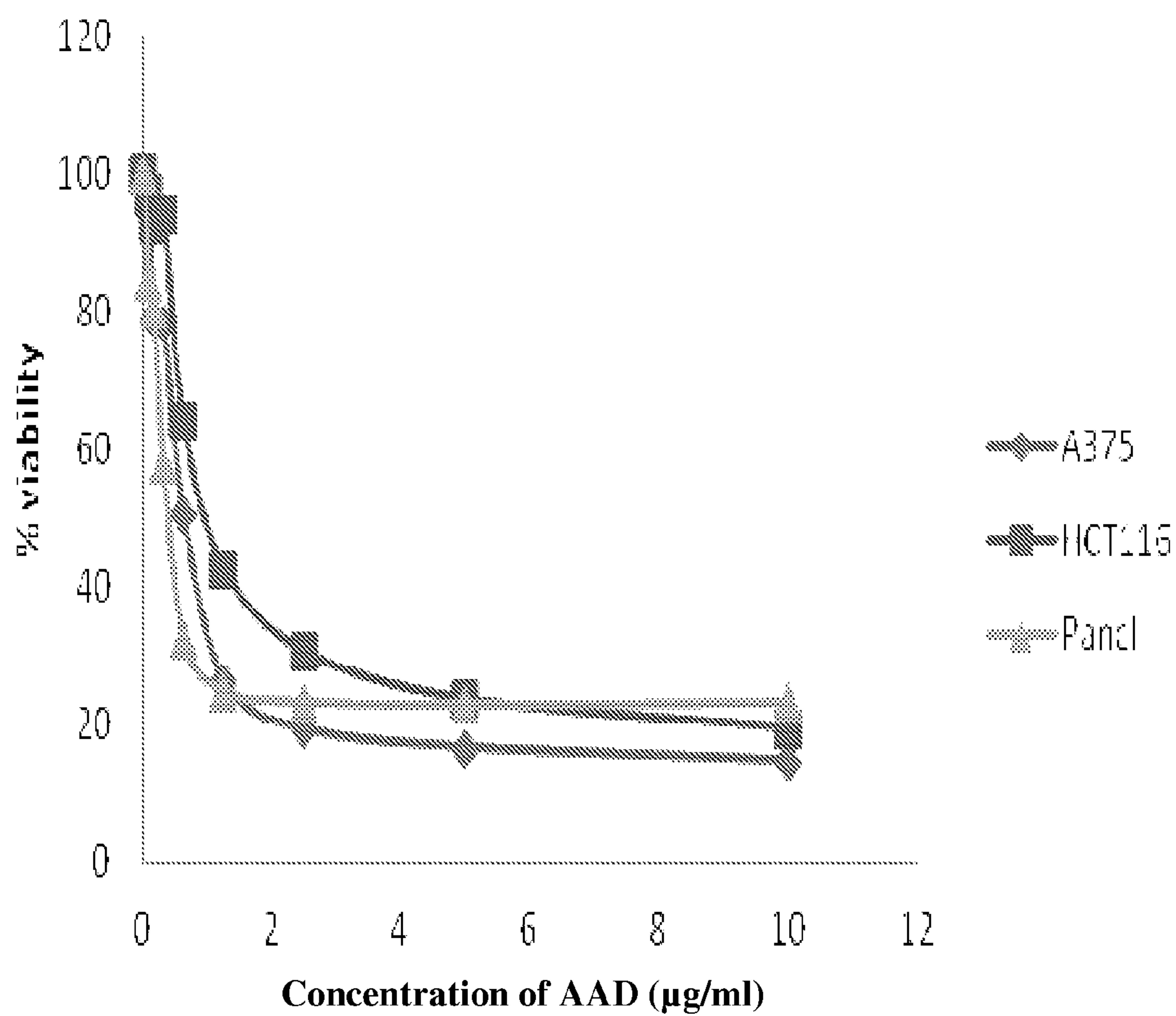


FIG. 9

20/22

(A)

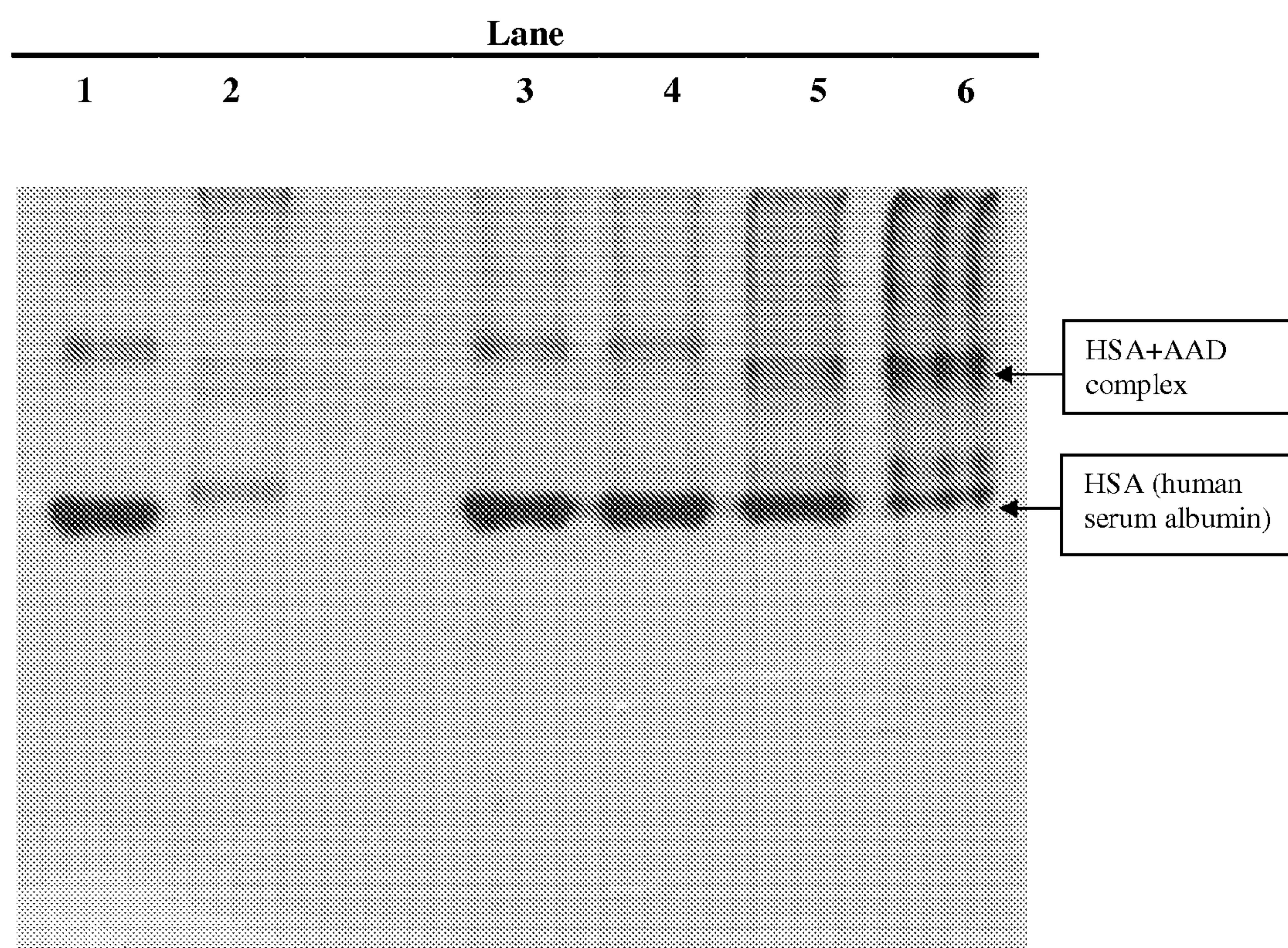


FIG. 10

(B)

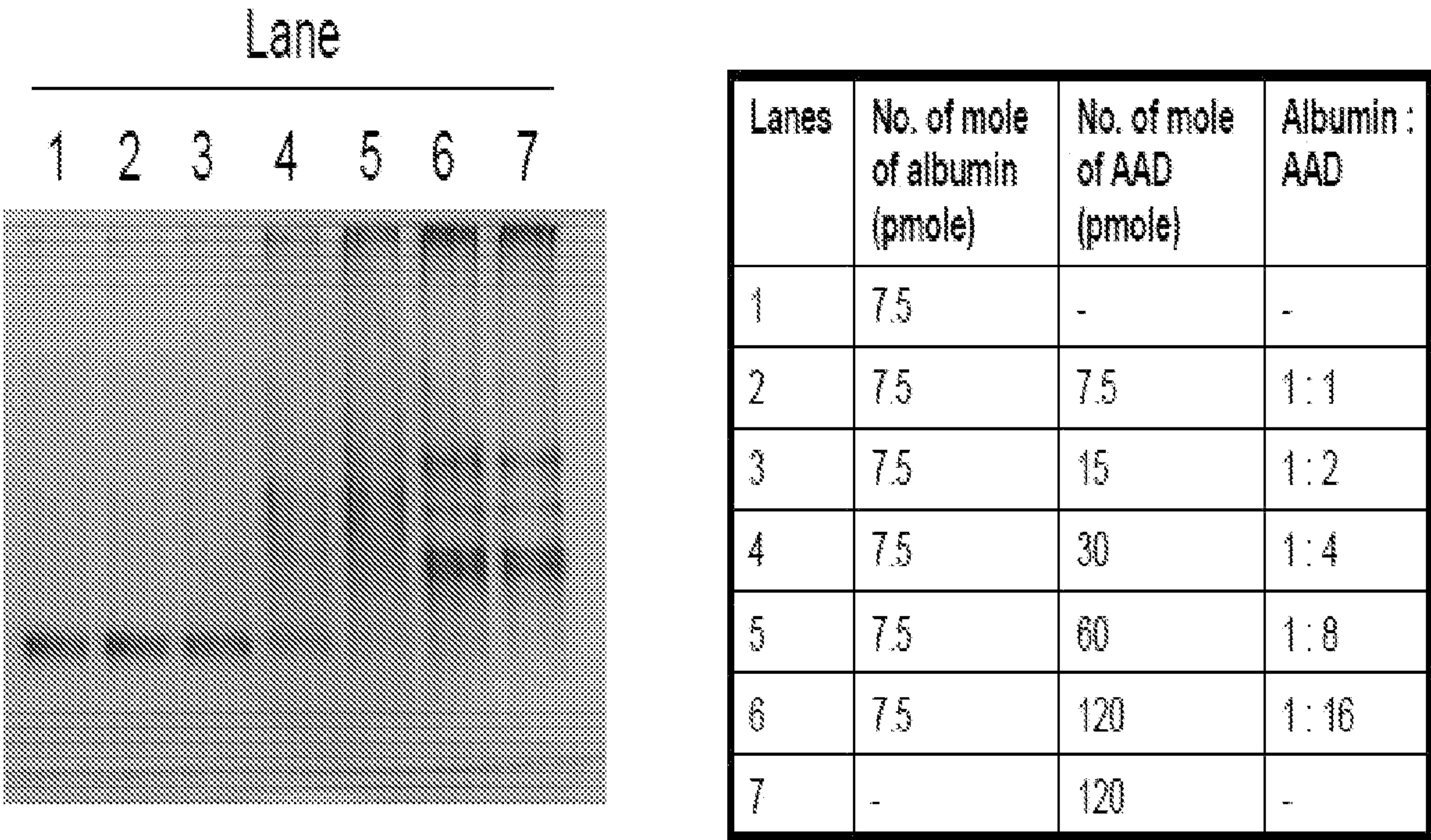
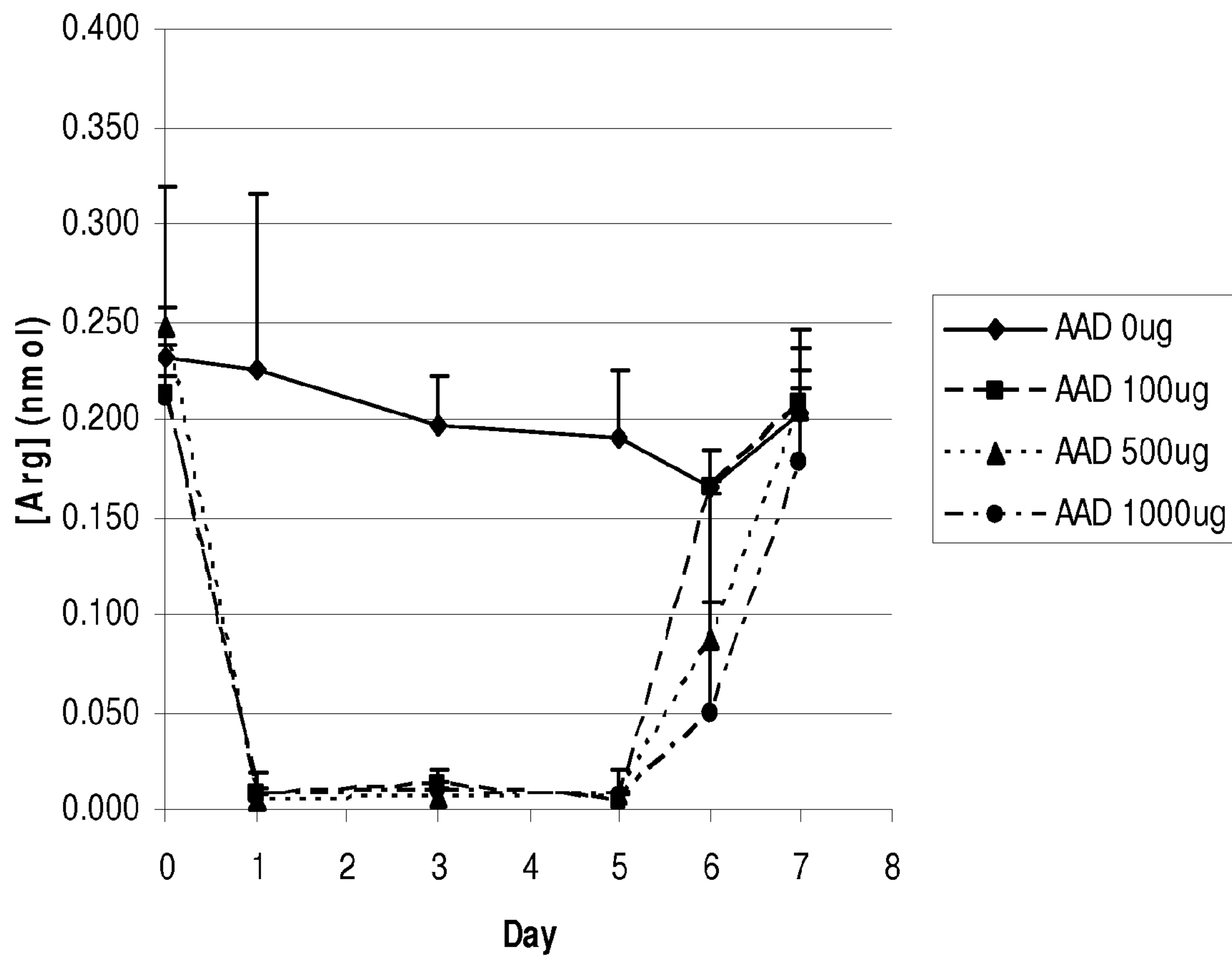


FIG. 10 (continued)

22/22

**Effect of AAD on mice plasma arginine levels (n=3)****FIG. 11**