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(54) Title: MODIFIED CHITIN BINDING DOMAIN AND USES THEREOF

(57) Abstract: Compositions and methods are provided for reversibly binding chitin binding domain (CBD) to a chitin or equivalent substrate under non denaturing conditions. CBD is modified preferably by a mutation to achieve this change in properties. In one embodiment, an aromatic amino acid residue located in the binding cleft of the CBD was altered resulting in reversible binding affinity for substrate in select conditions. Creating a modified CBD with an altered binding affinity for substrate provides new uses for CBD not previously possible with unmodified CBD which binds irreversibly to chitin.



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MODIFIED CHITIN BINDING DOMAIN AND USES THEREOF

BACKGROUND

5 The present invention relates to a modified chitin binding domain and methods for making the same where the modification alters the properties of the chitin binding domain so that it becomes capable, under select conditions, of elution from a substrate for which it has specific affinity.

10 Although a number of different approaches to protein purification exist, the application of recombinant techniques to generating fusion proteins from target proteins and substrate binding proteins (affinity tags) has provided efficient methods of separating target proteins from complex mixtures and/or large volumes. (LaVallie and McCoy, *Curr. Opin. Biotechnol.*, 6:501-506 (1995), U.S. Patent Nos. 5,834,247 and 5,643,758). Examples of substrate binding proteins include the chitin binding domain (CBD or ChBD) of chitinase which binds chitin substrate (U.S. Patent No. 5,837,247, Xu et al., *Methods Enzymol.* 326:376-418 (2000)), maltose binding protein which binds an amylose substrate (U.S. Patent No. 5,643,758), cellulose binding domain from cellulase which binds cellulose (U.S. Patent Nos. 5,962,289; 5,928,917; and 6,124,177) and His-Tag (an oligopeptide) which binds a Nickel charged column. (Van Dyke, et al. *Gene* 111:99-104 (1992)).
25 In addition to the above, Glutathione S-transferase (GST) bind

sepharose TM4B resin (Smith, D. B., and Johnson, K. S. *Gene* 67:31-40 (1998)).

Each of the above affinity tags has certain limitations.

5 For example, CBD irreversibly binds to chitin substrate and cannot be eluted under non-denaturing conditions. However, CBD represents a potentially useful affinity tag with widespread application since it is readily obtained from any of a family of enzymes identified as chitinases that are capable
10 of hydrolyzing chitin. As might be expected, chitinases are produced by a diverse range of organisms that either contain chitin or rely on chitin as a food source. These organisms include bacteria, fungi, plants and vertebrates. (Watanabe et al. *J. Bacteriol.*, 176:4465-4472 (1994), Jolles et al., *Chitin and Chitinases*, Birkhäuser Verlag, Basel (1999); Hashimoto et al.,
15 *J. Bacteriol.* 182:3045-3054 (2000)). CBD binds to chitin, a polysaccharide abundantly represented in nature. It is found in many fungal cell walls, nematode and insect exoskeletons, and crustacean shells.

20 Chitinases are characterized by a chitin binding domain (CBD) and a catalytic domain. For example, Chitinase A1 which is produced by *Bacillus circulans* WL-12 contains three discrete functional domains: an N-terminal family 18 catalytic domain, a
25 tandem repeat of fibronectin type III-like domains and a C-terminal chitin-binding domain (Figure 1) (Watanabe et al., *supra* (1994)). Moreover, since CBD is located within the chitinase at a site that is distinct from the catalytic domain, it

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naturally lacks hydrolytic activity when isolated from the enzyme for use as an affinity tag.

While CBD has a number of useful properties it lacks the property of reversible binding to chitin under non-denaturing conditions which limits its general utility.

SUMMARY OF THE INVENTION

In an embodiment of the invention, a protein is provided that includes a chitin binding domain (CBD) capable of reversibly binding a chitin substrate under selected non denaturing conditions. The chitin binding domain may be modified by having one or more mutated amino acids. The mutated amino acid may be an aromatic amino acid optionally positioned within a binding cleft of the CBD, for example, a tryptophan. In a particular embodiment, the tryptophan corresponds to Trp 687 of *B. circulans* chitinase A2.

Selected conditions for reversibly binding a chitin binding domain include a change in one of: ionic concentration, pH, detergent concentration, antagonist or agonist concentration. For example a change in ionic conditions may include a reduction in salt conditions.

In an additional embodiment of the invention, a method is provided for obtaining a chitin binding domain capable of reversible binding to a chitin substrate under non-denaturing

conditions where the method includes the steps of modifying at least one amino acid within the CBD, and determining whether the modified CBD is capable of reversibly binding chitin under selected conditions. One type of modification is a mutation of a portion of DNA sequence encoding the CBD followed by expression of the DNA in a host cell. The mutation may be introduced into the portion of the DNA sequence by substituting an existing oligonucleotide portion of the DNA sequence with an alternative oligonucleotide which differs in that it contains a mutation at a target site. For example, the target site may be a tryptophan located in the binding cleft of the CBD. When for example, the tryptophan is substituted with a phenylalanine, the CBD is capable of reversible binding to chitin under non-denaturing conditions. For example, non-denaturing conditions include a change in any of: ionic concentration; pH; detergent concentration; or antagonist or agonist concentration.

In an additional embodiment of the invention, a method is provided for producing and purifying a target protein molecule where the method includes the steps of: constructing a DNA expression vector which expresses a hybrid polypeptide in a transformed host cell, the hybrid polypeptide comprising the target protein molecule and a modified chitin binding domain where the chitin binding domain has a specific and reversible affinity for a substrate such as chitin or derivatives or analogues thereof; introducing the expression vector into an appropriate host cell;

expressing the hybrid polypeptide; contacting the hybrid polypeptide produced by the transformed cell with the substrate to which the CBD binds; and recovering the hybrid polypeptide. The hybrid polypeptide may for example be recovered from the substrate to which it is bound by altering the ionic condition or pH or by contacting the bound hybrid polypeptide with a detergent or an agonist or antagonist which displaces the hybrid polypeptide.

In an additional embodiment of the invention, a method is provided for purifying a chitin binding domain-target molecule conjugate from a mixture of molecules. The method includes the steps of adding to the mixture, a substrate having a specific and reversible affinity for CBD so as to permit binding and immobilizing of the conjugate to the substrate; removing the bound conjugate from the mixture; and eluting in altered ionic conditions, the conjugate from the substrate to obtain the purified conjugate.

In an additional embodiment of the invention, a kit is provided for purifying a recombinant protein, that includes a plasmid, the plasmid containing a DNA sequence encoding a modified CBD or portion thereof and an insertion site for inserting the DNA sequence encoding the recombinant protein; a substrate for specific and reversible binding of the fusion protein; and optionally a buffer for eluting the fusion protein from the substrate.

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In an embodiment of the invention, a vector within or separate from a host cell is provided where the vector is capable of expressing a modified chitin binding domain (CBD) fused to a protein molecule to be purified, the vector including a DNA fragment coding for the modified chitin binding domain or portion thereof, having a specific and reversible affinity for a substrate which binds to the chitin binding protein. The vector may further express an additional DNA fragment coding for the protein molecule to be purified where the additional DNA fragment is optionally located within or adjacent to the CBD sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Amino acid sequence alignment of the chitin-binding domain from *Bacillus circulans* chitinase A1 with domains of other procaryotic chitinases. Sequences were aligned with the program CLUSTAL V (40). Conserved residues are indicated in black boxes. Amino acids sequences shown are from: CBD of *Bacillus circulans* WL-12 chitinase A1 (*B. circulans* A1 (SEQ ID NO:1)), *Bacillus circulans* WL-12 chitinase D (*B. circulans* ChiD (SEQ ID NO:2)), *Aeromonas sp.* Strain 10S-24 chitinase II (*Aeromonas* ChiII (SEQ ID NO:3)), *Janthinobacterium lividum* chitinase (*J. lividum* Chitinase (SEQ ID NO:4)), *Serratia marcescens* 2170 chitinase C (*S. marcescens* ChiC (SEQ ID NO:5)), *Aeromonas sp.* Strain 10S-24 chitinase II (*Aeromonas* ChiII (SEQ ID NO:6)), *Aeromonas sp.* Strain 10S ORF1 (*Aeromonas* ORF1 (SEQ ID NO:7)), *Aeromonas*

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sp. Strain 10S-24 chitinase I (*Aeromonas* ChiI (SEQ ID NO:8)),
Serratia marcescens 2170 chitinase B (*S. marcescens* ChiB
(SEQ ID NO:9)), *Janthinobacterium lividum* chitinase (*J. lividum*
Chitinase (SEQ ID NO:10)), *Alteromonas* sp. Strain O-7
5 chitinase 85 (*Alteromonas* Chi85 (SEQ ID NO:11)),
Streptomyces griseus chitinase C (*S. griseus* ChiC (SEQ ID
NO:12)), *Vibrio harveyi* chitinase A (*V. harveyi* ChiA (SEQ ID
NO:13)), *Aeromonas caviae* extracellular chitinase A (*A. caviae*
ChiA (SEQ ID NO:14)). The first five sequences are considered
10 to belong to the CBD_{ChiA1} group. The number at the right of
each sequence represents the position of the last residue in
each sequence. The residues of CBD_{ChiA1} that were mutated,
are indicated by a star above the sequence. The numbers at
the top represent the position in *B. circulans* ChiA1.

15 Figure 2. Schematic ribbon drawing of the CBD of *Bacillus*
circulans chitinase A1.

Figure 2A. Drawing of the wild-type CBD of *Bacillus*
circulans chitinase A1.

20 Figure 2B. Drawing of the CBD harboring the W687F
mutation. β -strands are shown as curved arrows in yellow.
Secondary structure elements, N and C termini, and the
mutated residues are labeled. Red color represents
specifically the residue in position 687. The sequence of
25 *Bacillus circulans* chitinase A1 was obtained from NCBI
structures database and figures were obtained using Swiss-
Pdb Viewer version 3.7b1.

Figure 3. Chitin binding activity of PXB mutant proteins. The mutated CBD was expressed as a fusion protein (PXB, 56 kDa) consisting of the N-terminal paramyosin Δ Sal fragment (P, 26 kDa), the *Mxe* GyrA mini-intein (X, 22 kDa) and the mutated CBD of *Bacillus circulans* chitinase A1 (B, 8 kDa).

Figure 3A. A chitin binding assay for each PXB protein carrying an alanine substitution was carried out in Tris-buffer (pH 8) containing 50 mM NaCl.

Figure 3B. A chitin binding assays were performed in buffer containing either 50 mM NaCl (lanes 1-3) or 2 M NaCl (lanes 4-6) for the PXB mutant proteins carrying W687F.

Figure 3C. A chitin binding assay for W687Y in different NaCl conditions (50 mM NaCl in lanes 1-3 and 2M NaCl in lanes 4-6).

Figure 3D. A chitin binding assay for W687T in different NaCl conditions (50 mM NaCl in lanes 1-3 and 2M NaCl in lanes 4-6).

Figure 3E. A chitin binding assay for P689F in different NaCl conditions (50 mM NaCl in lanes 1-3 and 2M NaCl in lanes 4-6).

The samples were analyzed by Coomassie Blue stained SDS-PAGE. The mutated amino acid and its position in the CBD are indicated on top of each gel. UI, uninduced cell extract. Lanes 1 and 4, clarified cell extract. Lanes 2 and 4, chitin flow-through. Lanes 3 and 6, a sample of chitin beads following wash with the same buffer used for loading. Broad Range protein marker (kDa) is indicated on the left side of each gel.

Figure 4A. Characterization of the W687F mutant.

Chitin binding activity of PXB W687F mutant protein at various NaCl concentrations. Induced cells were lysed in 20 mM Tris-buffer (pH 8) containing various NaCl concentrations indicated at the top of the gel. The samples were analyzed by Coomassie Blue stained SDS-PAGE. UI, uninduced cell extract. Lane 1, crude cell extract. Lane 2, flow-through. Lane 3, a sample of chitin beads following wash with the appropriate buffer. Lanes 4 and 5, a fraction after elution with buffer containing 50 mM or no NaCl following loading and washing with buffer containing 2 M NaCl. Lane 6, a sample after passage of the eluted fraction adjusted to 2 M NaCl over a new chitin resin. Lane 7, a sample of chitin beads after reloading and washing with a buffer containing 2 M NaCl.

Figure 4B. Elution curves of PXB W687F mutant at different salt concentrations. Loading of the wild type PXB protein and of the W687F mutant was carried out at 2 M NaCl. For the PXB W687F mutant, elutions were achieved in 20 mM Tris (pH 8) containing either 50 mM NaCl (♦), 0.1 M NaCl (□), 0.5 M NaCl (▲), or 1 M NaCl (○). The elution of the wild type PXB was performed at 50 mM NaCl (×).

Figure 5. Purification of recombinant proteins fused to the ChBD carrying the W687F mutation.

Figure 5A. Schematic overview of the one-step affinity purification for recombinant proteins fused to the CBD (W687F).

Figure 5B. Purification of hGM-CSF-ChBD.

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Figure 5C. Purification of Her-2(KD)-ChBD.

The samples were analyzed by Coomassie Blue stained SDS-PAGE. Lane 1, uninduced cell extract. Lane 2, crude cell extract. Lane 3, supernatant from the crude cell extract after centrifugation. Lane 4, load of renatured proteins in 20 mM Tris buffer (pH 8) containing 2 M NaCl. Lane 5, renatured proteins flow-through. Lane 6, a sample of chitin beads after loading renatured proteins. Lane 7, eluted protein from chitin beads with 20 mM Tris-buffer (pH 8) containing 50 mM NaCl.

DETAILED DESCRIPTION OF THE EMBODIMENTS

The utility of CBD has been enhanced by modifications to the protein that cause the CBD to be capable of reversible binding to a chitin substrate under conditions that do not denature proteins (non-denaturing conditions).

The modified CBD may be linked to a protein or other molecule of interest whether covalently or by affinity binding or via a linker molecule to form a molecular conjugate. Where the conjugate is present in a mixture, it may be selectively bound to a CBD-specific substrate and eluted from the substrate under select conditions.

"Chitin binding domain", "CBD" or ChBD here refers to any binding domain derived from a naturally occurring or recombinant chitinase, including chitinases for which sequences are available from sequence databases such as

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GenBank to or contained within gene libraries made according to standard molecular biology techniques (Sambrook et al., Molecular Cloning: A Laboratory Manual, CSH 1989) using conserved sequence motifs such as described in Figure 1.

5 Figure 1 provides an example of sequence motifs observed when 13 chitin binding domains from different sources were aligned. Examples of chitinases include at least 6 different chitinases, A1, A2, B1, B2, C, and D present in *Bacillus*
10 *circulans* WL-12 (Watanabe et al., *J. Bacteriol.*, 172:4017-4022 (1990); Watanabe et al., *J. Bacteriol.*, 176:4465-4472 (1994)) derived from four genes, *chiA*, *chiB*, *chiC*, and *chiD*, with chitinase A2 and B2 being generated by proteolytic modification of chitinase A1 and B1, respectively (Alam et al., *J. Ferment. Bioeng.*, 82:28-36 (1996)).

15 A "target molecule" is a molecule of interest that includes any prokaryotic or eukaryotic, simple or conjugated protein that can be expressed by a vector in a transformed host cell and further includes proteins that may be subject to
20 post translational modifications in natural or synthetic reactions.

The term "protein" is intended to include peptides and derivatives of proteins or peptides and to further include
25 portions or fragments of proteins and peptides. Examples of proteins include enzymes including endonucleases, methylases, oxidoreductases, transferases, hydrolases, lyases, isomerases or ligases, storage proteins, such as

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ferritin or ovalbumin; transport proteins, such as hemoglobin, serum albumin or ceruloplasmin; structural proteins that function in contractile and motile systems, for instance, actin, myosin, fibrous proteins, collagen, elastin, alpha-keratin, glyco-proteins, virus-proteins and muco-proteins; immunological proteins such as antigens or antigenic determinants which can be used in the preparation of vaccines or diagnostic reagents; blood proteins such as thrombin and fibrinogen; binding proteins, such as antibodies or immunoglobulins that bind to and thus neutralize antigens; hormones and factors such as human growth hormone, somatostatin, prolactin, estrone, progesterone, melanocyte, thyrotropin, calcitonin, gonadotropin, insulin, interleukin 1, intereukin 2, colony stimulating factor, macrophage-activating factor and interferon; toxic proteins, such as ricin from castor bean or grossypin from cotton linseed and synthetic proteins or peptides.

It is further envisioned that in addition to a protein expressed by a vector, the target molecule may alternately be a non-protein, non-substrate molecule isolated from nature or made synthetically which may form a conjugate with CBD by means of covalent linkage subsequent to synthesis or by non-covalent linkage (such as affinity binding). A target molecule also refers to a molecule which may bind to a protein complex formed between the affinity tag and a protein where the affinity tag binds to a substrate. The molecule may be any of: an organic molecule or an

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inorganic molecule including a co-factor, ligand, protein, carbohydrate, lipid, synthetic molecule, ion, where the inorganic molecule further includes fluorophors and dyes or mixtures of any of the above.

5

"Substrate" refers to any molecule to which CBD will bind. This preferably includes chitin which is an insoluble β -1,4-linked homopolymer of *N*-acetyl-D-glucosamine, P. Jolles et al. *Chitin and chitinases*, Birkhäuser Verlag (1999), Basel., but may also include chitin analogues and derivatives that are naturally occurring or prepared in part or wholly by chemical synthesis. The substrate may be formed into beads, colloids, columns, films, sponges, filters, coating or other suitable surfaces for use in binding an affinity tag for purposes that include isolation or purification of a target molecule or analysis of the presence or amount of a target molecule in a diagnostic test format or for binding a marker as an indicator of a chemical reaction or other use.

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"Modified CBD" refers to any change to a CBD that results in its binding to substrate being altered under select conditions where such alteration in binding would not occur in unmodified CBD.

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"Selected condition(s)" refers to any condition which when applied to a conjugate of a target molecule and CBD bound to substrate via the CBD, causes reversal of binding.

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The selected condition is preferably required to be of the type that does not degrade the target molecule.

"Host cells" refers to cells that express target molecules, CBD and/or fusion proteins and include any known expression system in prokaryotes or eukaryotes including bacterial host cells, yeast, invertebrate, fish and mammalian cells including human cells

Desirable features of a modified CBD

Desirable features of a modified CBD may include any or all of the following:

(a) a size or other characteristics of the modified CBD should preferably not interfere with the function of the target protein to which it is associated. An advantage of non-interference is that cleavage of the CBD from the protein is not required to obtain purified active target protein. Indeed, where the size of the affinity tag is less than about 30kb or less than 20kd, interference with functionality of the target protein may be minimized or avoided. Examples VII-IX show that CBD (about 6Kd) does not interfere with the function of a target recombinant protein EK-CBD. In those circumstances where the target protein is very small, a DNA sequence encoding a linking peptide sequence may be inserted between DNA for the affinity tag and the target peptide. The resultant fusion protein may be cleaved by a proteolytic agent

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to liberate the target protein after purification has been completed;

5 (b) an ability of the modified CBD to bind tightly to both a substrate and the target protein under one set of conditions and under a separate set of conditions, to maintain an association with the target protein while being eluted from the substrate. Example V shows how the binding of modified CBD to substrate can be made energetically less favorable
10 under selected conditions by introducing mutations into the protein;

(c) an ability to recognize a substrate that is readily available in nature or capable of cost effective manufacture
15 and may be formed into any of a variety of formats according to the desired use such as beads or columns for purification of a target molecules. Figure 5A shows results of a one step chitin column purification of hGM-CSF-CBD and Her-2(KD)-CBD (Figure 5B and 5C);

20 (d) Absence of properties that cause degradation of the substrate by the modified CBD or target protein under the set of conditions in which the substrate is used to separate the target protein from a mixture. For example, the CBD lacks
25 hydrolytic activity associated with chitinase where chitinase digests chitin.

Identifying a Suitable Modification of the chitin binding domain
by targeted mutation of the DNA sequence encoding the CBD

5 Mutations in DNA which result in an altered amino acid
sequence may be random or may be targeted to a specific
amino acid or amino acids. One criteria for selecting an amino
acid target is the location of the amino acid in the protein as
determined by crystallographic data. For example, a targeted
amino acid may be located on the surface of the CBD or within
10 the binding cleft.

In one embodiment of the invention, targeted
mutagenesis results in changes to one or more amino acids in
the 45 residues CBD selected according to the tertiary
15 structure of the protein. As shown in Figure 2, CBD has a
compact and globular structure containing two antiparallel β -
sheets and a core region formed by hydrophobic and aromatic
residues. Several residues that may be important for the
hydrophobic interaction with chitin may have one or more of
20 the following properties: (i) they are well conserved among
different bacterial chitinases; (ii) they exist on the surface of
the molecule or in the hydrophobic core, and (iii) they are
hydrophobic or aromatic amino acids with the potential to
form hydrophobic interactions with chitin. For example, Trp⁶⁸⁷,
25 Pro⁶⁸⁹ and Pro⁶⁹³, are highly conserved among different
bacterial chitinases and exist on the surface of the molecule
with the potential to form hydrophobic interactions with chitin.

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Using methods of targeted mutagenesis, such as described in Example I, any desired amino acid can be altered and the effect on binding of CBD to chitin measured under selected conditions. While the method of targeted mutagenesis using mutagenesis linkers is effective, there are many alternative approaches known to one of ordinary skill in the art for targeted mutagenesis which may alternatively be used to modify CBD. In Example I, mutations of amino acids at positions 681, 682 and 687 in the protein are described. However, the method of Example I could also be applied to mutating any other amino acid in the CBD. Once an altered CBD has been formed, it may be assayed according to Example II in order to determine whether the CBD is capable of reversible binding to its substrate.

In Example I, a mutant CBD with an altered amino acid at position 687 was found to be capable of reversible binding to chitin when the native Tryptophan which is a hydrophobic residue within the binding cleft of CBD was replaced with phenylalanine. This finding however does not preclude other amino acid substitutions at this location being effective although substitution with alanine, tyrosine or threonine, appeared to completely abolish CBD binding to chitin (Figure 3). Nor does this finding preclude amino acid substitution at other locations in the protein. While not wishing to be bound by theory, it appears from the 3D structure of the CBD, that Trp687 lies in the binding surface formed between the two β -sheets and interacts directly with the chitin chain through

hydrophobic interactions (Figure 2) presumably involving aromatic ring polarization. Hence, while mutation of Trp⁶⁸⁷ to phenylalanine still permitted binding at 2 M NaCl where the benzene ring of phenylalanine residue substituted for the indole ring of tryptophan, affinity of binding of CBD to chitin became altered so as to be responsive to altered ionic strength. In contrast, replacement of Trp⁶⁸⁷ with tyrosine abolished binding to the chitin substrate probably due to the presence of a hydroxy-group on the phenyl ring that may interfere with the general hydrophobicity of the region.

Interestingly, mutation of Pro689 to phenylalanine in the CBD abolished binding to chitin while mutation to alanine showed no effect (Figures 3A and 3E). Pro689 is positioned in the loop between β_4 and β_5 in close proximity of Trp687. Due to the cyclic and rigid nature of its pyrrolidine side group, introduction of phenylalanine in this position probably disturbs the positioning of Trp687 in the structure. Our results also indicate that single amino acid substitutions of Trp656 or Trp696 have no substantial effect on the chitin binding activity.

While hydrophobic amino acids have been initially targeted by mutagenesis, the findings do not preclude the possibility that non-hydrophobic amino acids in the CBD may be modified to provide reversible binding of CBD to chitin under select conditions. Moreover, while Example I describes a particular mutation in the CBD of *Bacillus circulans* chitinase

A1, it is expected, based on evolution of CBD as a class, that modification to a targeted amino acid in a CBD from one source will cause a similar effect in CBDs in general.

5 Desirable modifications of CBD provide a high affinity of binding to chitin under one set of conditions with reversible binding under altered conditions. For example the altered conditions may be a shift in ionic strength of the elution buffer from one ionic strength to a greater or lesser ionic strength.

10 For example, ionic strength may be altered by modifying the NaCl concentration in the buffer. For example, whereas modified CBD may bind irreversibly in a buffer having a salt concentration, in the range of 0.2 M - 3M, the modified CBD may be eluted when the salt concentration is altered to 0.1 -

15 1M NaCl. While the above ranges overlap, it should be understood that it is intended that different concentrations of NaCl in buffer determine whether binding of CBD to substrate is reversible or non-reversible. Example III describes how in a buffer containing 2M NaCl, a modified CBD binds strongly to

20 chitin while at a different salt concentration (50 mM NaCl), the affinity for CBD for substrate is reduced permitting elution of CBD from the substrate (Example III and IV). Alternatively, instead of changing salt conditions, pH may be changed to cause modified CBD to be reversibly bound to substrate. For

25 example, CBD having a Try 687 modified to phenylalanine binds strongly to chitin at a pH in the range of 6 to 11. However, no or very poor binding of the CBD to chitin occurs at or below pH 5. Other selected conditions may include a

change in temperature, change in detergent concentration or addition or removal of competitive binding molecules such as agonists or antagonists for example, oligopolysaccharide homologs of chitin or CBD analogs.

5

The formation of a conjugate of CBD with a target molecule may include either a covalent or non-covalent association between the component molecules. There are many methods known in the art for creating a conjugate. If the target molecule is a protein, the protein may be covalently linked to the CBD during recombinant synthesis in a host cell. Accordingly, the DNA sequence corresponding to CBD or target protein may be contained within a plasmid or chromosomal DNA in a host cell for expression of a fusion protein. In certain circumstances, the target protein may become covalently linked to the CBD after cleavage of an intein or alternatively a target protein may be linked to a CBD post translationally by protein ligation or by other means (U.S. Patent No. 5,834,247; International Publication No. WO 00/47751 and WO 01/57183).

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Genes coding for the various types of protein molecules including those described below may be obtained from a variety of prokaryotic or eukaryotic sources, such as plant or animal cells or bacteria cells. The genes can be isolated from the chromosomal material of these cells or from plasmids of prokaryotic cells by employing standard, well-known techniques. A variety of naturally occurring and synthetic

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plasmids having genes encoding many different protein molecules are now commercially available from a variety of sources. The desired DNA also can be produced from mRNA by using the enzyme reverse transcriptase.

5

Preparation of DNA fusion and expression vectors may be achieved as described in the art (U.S. Patent No. 5,643,748) or as described in Example I or by other means known in the art. For example, the following protocol may be followed:

10

I. Preparation of Fusion Vector

A) The DNA encoding for the desired binding protein is purified.

15

B) The DNA is inserted into a cloning vector such as pBR322 and the mixture is used to transform an appropriate host such as *E. coli*.

C) The transformants are selected, such as with antibiotic selection or auxotrophic selection.

20

D) The plasmid DNA is prepared from the selected transformants.

E) The binding activity domain of the protein is determined and convenient restriction endonuclease sites are identified by mapping or created by standard genetic engineering methods.

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II. Insertion of DNA Coding for the Protein Molecule into the Fusion Vector

- 5 A) The protein molecule gene is cloned by standard genetic engineering methods.
- B) The protein molecule gene is characterized, e.g. by restriction mapping.
- C) A DNA restriction fragment which encodes the protein molecule is prepared.
- 10 D) The protein molecule DNA fragment is inserted in the binding protein fusion vector so that an in-frame protein fusion is formed between the DNA fragment coding for the modified CBD and the DNA fragment coding for the protein molecule.
- 15 E) The vector containing this hybrid DNA molecule is introduced into an appropriate host.

III. Expression and Purification of the Hybrid Polypeptide

- 20 A) The host cell containing the fusion vector is cultured.
- B) Expression of the fused gene is induced by conventional techniques.
- 25 C) A cell extract containing the expressed fused polypeptide is prepared.
- D) The hybrid polypeptide is separated from other cell constituents using an affinity column having as a

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matrix a substance to which the modified CBD part of the hybrid polypeptide has a specific affinity.

E) The bound purified hybrid polypeptide can be recovered and/or utilized by the following methods:

- (1) if the protein molecule's biological activity is maintained in its hybrid or fused configuration it may recovered from the column by eluting under selected conditions and used directly after elution in its hybrid form;
- (2) the protein molecule may be separated from the modified CBD either before or after elution from the column by proteolytic or chemical cleavage; and
- (3) the column may be used as a bioreactor with the fusion protein immobilized on the column, e.g. by contacting and reacting the bound fusion protein with a substrate which interacts with the biologically active portion of the protein molecule.

Linking Sequence

A DNA fragment coding for a predetermined peptide may be employed to link the DNA fragments coding for the binding protein and protein molecule. The predetermined peptide is preferably one which recognized and cleaved by a proteolytic agent such that it cuts the hybrid polypeptide at or near the protein molecule without interfering with the biological activity

of the protein molecule. One such DNA fragment coding for a predetermined polypeptide is described in Nagai et al., *Nature* 309:810-812 (1984). This DNA fragment has the oligonucleotide sequence: ATCGAGGGTAGG (SEQ ID NO:15) and codes for the polypeptide Ile-Glu-Gly-Arg (SEQ ID NO:16). This polypeptide is cleaved at the carboxy side of the arginine residue using blood coagulation Factor Xa. As noted above the linking sequence, in addition to providing a convenient cut site if such is required, may also serve as a polylinker, i.e. by providing multiple restriction sites to facilitate fusion of the DNA fragments coding for the target and binding proteins, and/or as a spacing means which separates the target and binding protein which, for example, allows access by the proteolytic agent to cleave the hybrid polypeptide. Other examples of linkers include GATGACGATGACAAG (SEQ ID NO:45) coding for Asp-Asp-Asp-Asp-Lys (SEQ ID NO:46) which is cleaved by enterokinase I and CCGGGTGCGGCACACTCAC (SEQ ID NO:47) coding for Pro-Gly-Ala-Ala-His-Tyr (SEQ ID NO:48) which is cleaved by Genenase I (New England Biolabs 2002/2003 Catalog, page 163; Beverly, MA). Other linkers not generally cleaved by a protease include a polyasparagine linker which consists of 10 Asp amino acids and is encoded by AACAAACAACAACAACAACAACAACAACAACAACAAC (SEQ ID NO:49) and a "kinker" linker from M13 gene 3 protein with a Gly-Gly-Ser-Gly sequence.

The formation of a conjugate of modified CBD with the target molecule provides special advantages in purifying

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target molecules on a large scale or small scale. In Examples, VI-IX, the target molecule was expressed in host cells as a fusion protein with modified CBD. The fusion protein whether present in the production media or associated with the host cells which may be disrupted after harvesting, becomes immobilized by binding to substrate. After removal of the unbound material, the substrate to which the fusion protein is bound is subjected to non-denaturing conditions such as a particular ionic concentration or pH causing the fusion protein to be released into a selected buffer. The insoluble substrate can then be removed by precipitation, filtration or other standard techniques for removal of particles from a solution. Where the CBD does not interfere with the function of the target protein, cleavage of the CBD from the target protein is not required.

The above approach finds application in the purification of secreted proteins in microbial fermentation. Whereas purification of secreted proteins have the advantage of avoiding breaking the host cells prior to recovery, the desired secreted proteins may be present in large volumes of growth media. Handling large volumes of growth media presents a set of problems for which a solution would be desirable. For example, the yeast *Kluyveromyces lactis* is an important organism for industrial scale production of proteins. For over a decade, *K. lactis* has been used for heterologous protein production in the food industry due to its ability to grow to high cell density and secrete large amounts of recombinant

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protein. A drawback to the protein secretion method is that typically large volumes of culture medium must be processed to obtain highly purified recombinant protein. To demonstrate one approach to this, we have shown how secreted bovine enterokinase-CBD fusion protein can be purified from batch harvests using a mutant version of the *Bacillus circulans* chitinase A1 CBD as an affinity tag (Examples VII-IX).

The ability to alter the chitin binding domain from chitinase, so as to make its binding to substrate reversible significantly enhances the utility of this protein for purification of target molecules from different environments including from simple or complex mixtures of molecules in small or large liquid volumes.

The present invention is further illustrated by the following Examples. These Examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

The references cited above and below are herein incorporated by reference.

EXAMPLE I

PLASMID CONSTRUCTION

The vector pPXB expresses a tripartite fusion protein consisting of the *Dirofilaria immitis* paramyosin $\Delta Sa/I$ fragment

(Steel et al., *J. Immunol.*, 145:3917-3923 (1990)) followed by the *Mxe* GyrA intein of *Mycobacterium xenopi* (*Mxe* intein) and the wild type CBD from *Bacillus circulans* WL-12 fused to the C-terminus of the intein (Evans et al., *Biopolymers* 51:333-342 (1999a)). The sequence encoding human granulocyte-macrophage colony-stimulating factor (hGM-CSF) (Cantrell et al., *Proc. Natl. Acad. Sci. USA*, 82:6250-6254 (1985); Mingsheng et al., *J. Biotechnol.* 1995:157-162 (1995)) was amplified by polymerase chain reaction (PCR) using hGM-CSF cDNA (ATCC-39754) as template and the primers: 5'-CTC GAGCATATGGCACCCGCCCCGCTCGC-3' (SEQ ID NO:17) and 5'-CGTGGTTGCTCTTCCGCACTCCTGGACTGGCTCCCAGCAG-3' (SEQ ID NO:18). The resulting product was cloned into pTWIN1 vector (Evans et al., *J. Biol. Chem.*, 274:18359-18363 (1999b)) using *Nde*I and *Sap*I sites yielding pGM-CSF-XB. Expression of this construct produces hGM-CSF fused to the *Mxe* intein-CBD. A *Hind*III site was introduced into the CBD wild type sequence by silent base substitution. To do this, the intein-CBD coding region was amplified by PCR using Vent® DNA polymerase (New England Biolabs, Inc.; Beverly, MA) and the following primers 5'-AGATGCACTAGTTGCCCTAC-3' (SEQ ID NO:19) and 5'-TGTACGCTGCAGTTACAAGCTTGTGTGGGGCTGCAAACATTTAT-3' (SEQ ID NO:20). The resulting fragment was cloned in-frame in pPXB and pGM-CSF-XB vectors using the *Spe*I and *Pst*I sites thereby replacing the original CBD sequence by a CBD with a 16 amino acid deletion of its C-terminus. The following oligonucleotides and their appropriate complement were used to introduce the missing C-terminal residues into the *Hind*III

and *Pst*I sites in both vectors: W687F, 5'-AGCTTGGCAGGATTT
GAACCATCCAACGTTTCCTGCCTTGTGGCA GCTTCAATAACTGCA-3'
(SEQ ID NO:21); W687A/W696A, 5'-AGCTTGGCAGGAGCCGAA
CCATCCAACGTTTCCTGCCTTGGCCCAGCTTCAATAACTGCA-3' (SEQ
5 ID NO:22); W687F/W696F, 5'-AGCTTGGCAGGATTTGAACCACCA
ACGTTTCCTGCCTTGTTCAGCTTCAATAACTGCA-3' (SEQ ID
NO:23); W687T, 5'-AGCTTGGCAGGAACCGAACCATCCAACGTT
CTGCCTTGTGGCAGCTTCAATA ACTGCA-3' (SEQ ID NO:24);
W687Y, 5'-AGCTTGGCAGGATATGAACCATCCAACGTTTCCTGCCT
10 TGTGGCAG CTTCAATAACTGCA-3' (SEQ ID NO:25); P689A, 5'-
AGCTTGGCAGGATGGGAAGCCTCCAACGTTTCCTGCCTTGTGGCAGCT
TCAATAACTGCA-3' (SEQ ID NO:26); P689F, 5'-AGCTTGGCA
GGATGGGAATTTTCCAACGTTTCCTGCCTTGTGGCAGCTTCAATAACTG
CA-3' (SEQ ID NO:27); P693A, 5'-AGCTTGGCAGGATGGGAACCAT
15 CCAACGTTGCCGCCTTGTGGCAGCTTCAATAACTGCA-3' (SEQ ID
NO:28); P693F, 5'-AGCTTGGCAGGATGGGAACCATCCAACGTTGC
CTTGTGGCAGCTTCAATAACTGCA-3' (SEQ ID NO:29); W696F, 5'-
AGCTTGGC AGGATGGGAACCATCCAACGTTTCCTGCCTTGTTCAGCT
TCAATAACTGCA-3' (SEQ ID NO:30). The mutagenesis linkers
20 were formed by annealing appropriate complementary
oligonucleotides. Other CBD mutations were introduced into
pGM-CSF-XB vector by linker replacement using the *Age*I and
*Mfe*I sites and the following oligonucleotides and their
appropriate complement: W656A, 5'-CCGGTCTGAACTCAGGC
25 CTCACGACAAATCCTGGTGTATCCGCTGCCCAGGTCAACACAG
CTTATACTGCGGGAC-3' (SEQ ID NO:31); W656F, 5'-CCGGTCT
GAACTCAGGCCTCACGACAAATCCTGGTGTATCCGCTTTTCAGGTC
AACACAGCTTATACTGCGGGAC-3' (SEQ ID NO:32). Mutations in

position 681 and 682 into *MfeI* and *HindIII* sites were achieved using the following oligonucleotides and their complement: H681A, 5'-AATTGGTCACATATAACGGCAAGACGTAT
AAATGTTTGCAGCCCGCCACA-3' (SEQ ID NO:33); H681F, 5'-AA
5 TTGGTCACATATAACGGCAAGACGTATAAATGTTTGCAGCCCTTTACA
-3' (SEQ ID NO:34); T682A, 5'-AATTGGTCACATATAACGGCAAGA
CGTATAAATGTTTGCAGCCCCACGCA-3' (SEQ ID NO:35). pGM-
CSF-XB constructs containing mutations were transferred into
pPXB using *SpeI* and *HindIII* sites. pGM-CSF-CBD vector was
10 constructed by replacing the *Mxe* GyrA intein coding region in
pGM-CSF-XB plasmid carrying the W687F mutation by a short
linker using the *SpeI* and *AgeI* sites and the following
oligonucleotides and their appropriate complements: 5'-CTA
GTGCCCCGGGCCAA-3' (SEQ ID NO:36). pCBD was constructed
15 by replacement of the sequence coding for the paramyosin
and the *Mxe* GyrA intein in pPXB carrying the W687F mutation
with a polylinker using the following oligonucleotide and its
appropriate complement: 5'-AGCTTGGCAGGATATGAACCA
TCCAACGTTCTGCCTTGTGGCAGCTTCAATAACTGCA-3' (SEQ ID
20 NO:37). The polylinker region permits cloning of a gene of
interest in-frame to the mutated CBD. The sequence encoding
the kinase domain of Her-2 [Her-2(KD)] (Yamamoto et al.,
Nature 319:230-234 (1986)); Doherty et al., *Proc. Natl. Acad.*
Sci. USA 96:10869-10874 (1999)) was amplified by PCR using
25 Human Heart Marathon Ready cDNA (Clontech; Palo Alto, CA)
and the following primers: 5'-GGCTCTTCATGCGGAGACTG
CTGCAGGAAACGGAG-3' (SEQ ID NO:38) and 5'-GGCTCTTC
CGCCGCCCTGCTGGGGTACCAGATACTCCTC-3' (SEQ ID NO:39).

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The resulting product was cloned into pCBD using the *SapI* site yielding pHer-2(KD)-CBD vector. pHer-2(KD)-CBD expresses a two-part fusion protein consisting of the cytoplasmic kinase domain of the human Her-2 protein [Her-2(KD)] fused at its C-terminus to the CBD harboring the W687F mutation.

EXAMPLE II

IN VITRO CHITIN-BINDING ASSAY

Escherichia coli strain ER2566 (New England Biolabs, Beverly, MA; Chong et al., *Gene* 192:271-281 (1997)), harboring pPXB or its mutant derivatives, was grown at 37°C in 1 liter of LB medium containing 100 µg/ml of ampicillin to an A_{600} of 0.5-0.7. The culture was induced with 0.3 mM isopropyl-β-D-thiogalactoside (IPTG) at 30°C for 3 hours or at 16°C overnight under the control of the T7 promoter (Studier et al., *Methods Enzymol.*, 185:60-89 (1990)). The proteins expressed from pPXB are referred to as PXB fusion proteins in our study. The binding assay was carried out by resuspension of the cell pellet in 20 mM Tris-HCl (pH 8) containing 2 M or 50 mM NaCl. Following sonication of the resuspended cell pellet, debris was removed by centrifugation at 4,000 x *g* for 30 minutes. Clarified supernatants were loaded at 4°C onto a column with a 3 ml bed volume of beads made of insoluble chitin (New England Biolabs, Beverly, MA) previously equilibrated in the same buffer as that used for resuspension. Equivalent amounts of load, flow-through, and a sample of

chitin beads for each of the NaCl concentrations were analysed by electrophoresing a 12% Tris-glycine gel (Invitrogen, Carlsbad, CA) and staining with Coomassie Brilliant Blue for visualisation.

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EXAMPLE III

ASSAY FOR NaCl-DEPENDENT CHITIN-BINDING AND ELUTION

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Expression of the PXB (W687F) fusion protein was conducted as described above. Binding of the PXB (W687F) mutant protein to chitin was carried out in 20 mM Tris-HCl (pH 8) containing either 2, 1, 0.5 or 0.05 M NaCl. Appropriate buffer was used for resuspension of the cell pellet and wash of chitin resin after loading. For the elution assay, resuspension of cell pellet and sonication were performed using the 20 mM Tris-HCl (pH 8) buffer containing 2 M NaCl. After the centrifugation step of the cell extract, the supernatant was loaded onto a chitin column previously equilibrated with buffer containing 20 mM Tris-HCl (pH 8) and 2 M NaCl. The PXB protein was eluted with 20 mM Tris-HCl (pH 8) buffer containing either 1 M, 0.5 M, 0.1 M or 50 mM NaCl. Thirty 1-ml fractions were collected. NaCl concentration of the 50 mM NaCl-eluted fraction was shifted back to 2 M in order to test whether binding was a reversible phenomenon. Recombinant proteins for the NaCl-dependent chitin binding and elution assay were subjected to SDS-PAGE analysis on 12% Tris-glycine gel and the protein concentration was

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determined by Bradford assay (Bradford, *Anal. Biochem.*, 72:248-254 (1976)) (Biorad, Cambridge, MA).

EXAMPLE IV

EFFECT OF CBD MUTATIONS ON BINDING TO INSOLUBLE CHITIN

In order to investigate the contribution of highly conserved residues of chitinase A1 CBD to chitin binding activity, single alanine substitutions were constructed in a 56 kDa fusion protein PXB possessing a C-terminal CBD as summarized in Table 1. The binding activities of the alanine replacement mutants were assayed by passage of the clarified induced cell extract over chitin resin in a buffer containing 50 mM NaCl. SDS-PAGE was used to examine the binding efficiency by comparing the load to the flow-through. In addition, a fraction of chitin resin was also subjected to SDS-PAGE analyses after extensive washing of the column. As shown in Figure 3, all alanine mutant proteins, except the W687A and the W687A/W696A double mutants, bound efficiently to chitin resin as indicated by the absorption of most PXB by the chitin resin after passage over the column (lane 2) and the presence of PXB species in the chitin resin fraction following a wash step (lane 3). In contrast, the W687A and the W687A/W696A double substitutions abolished the chitin binding activity since the amount of PXB species was not significantly reduced in the cell extract after passage over the column and was not present in the chitin

Table 1

Characterization of chitin-binding domain mutants		
Mutants	Percentage of chitin binding in various NaCl concentration	
	2 M	0.05 M
WT	>90	>90
W ⁶⁵⁶ A	>90	>90
H ⁶⁸¹ A	>90	>90
T ⁶⁸² A	>90	>90
W ⁶⁸⁷ A	<10	<10
E ⁶⁸⁸ A	>90	>90
P ⁶⁸⁹ A	>80	>90
P ⁶⁹³ A	>80	>80
W ⁶⁹⁶ A	>80	>90
W ⁶⁸⁷ A/W ⁶⁹⁶ A	<10	<10
W ⁶⁵⁶ F	>90	>90
H ⁶⁸¹ F	>90	>90
W ⁶⁸⁷ F	>90	<10
W ⁶⁸⁷ T	<10	<10
W ⁶⁸⁷ Y	<10	<10
P ⁶⁸⁹ F	<10	<10
P ⁶⁹³ F	>90	>90
W ⁶⁹⁶ F	>60	>90
W ⁶⁸⁷ F/W ⁶⁹⁶ F	>80	<10
E ⁶⁸⁸ Q	>90	>90

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resin fraction (W687A and W687A/W696A, lanes 2 and 3).

Furthermore, the same pattern of binding was obtained when binding assays were conducted with a 20 mM Tris-HCl buffer containing 2 M NaCl (data not shown). Therefore, the data suggested that W687 plays an important role in the interaction between CBD and chitin.

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Based on structural modeling (Figure 2), we reasoned that a conservative replacement by a hydrophobic and

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aromatic residue such as phenylalanine might compensate and mimic the role of W687. When the binding assay was performed in a buffer containing 50 mM NaCl, it appeared that the binding profile for the PXB (W687F) mutant protein (lanes 2 and 3, Figure 3B) was similar to that of the alanine substitution mutant. The binding assay was further performed in a buffer containing 2 M NaCl to assess whether interaction of the mutant proteins to chitin could be affected by ionic strength since higher salt concentration might enhance hydrophobic interaction and therefore increase the binding efficiency of the CBD to chitin. Under high salt conditions, the PXB protein harboring the W687F mutation (lanes 5 and 6, Figure 3B) was functionally active and bound chitin as indicated by a significant decrease of the PXB (W687F) species in the flow-through (lane 5) and its presence in the chitin resin fraction (lane 6). The results suggested that binding activity can be restored by the introduction of a phenylalanine residue at position 687 with a concomitant increase in NaCl concentration. On the other hand, replacement of W687 with tyrosine appeared to interfere with binding to chitin possibly due to an additional hydroxy group on the phenyl ring. The binding of the W687Y mutant was noticeably decreased in the reaction mixture containing either 50 mM or 2 M NaCl (Figure 3C). The low affinity of this PXB species for chitin was evident since most of the mutant protein remained in the extract after passage over chitin resin at both 50 mM and 2 M NaCl concentration (lanes 2 and 5) and was absent in the sample of chitin beads (lanes 3 and 6).

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Furthermore, replacement of Trp687 by a threonine residue also resulted in a failure to bind chitin (Figure 3D).

Mutation of other hydrophobic and aromatic residues, W656, H681, P693 and W696 to phenylalanine did not significantly affect the affinity to chitin in either 2 M or 50 mM NaCl (data not shown). However, introduction of a phenylalanine residue in place of Pro689 caused a substantial decrease in the binding efficiency (Figure 3E) compared to the P689A mutation, resulting in an increase of the mutant protein in chitin flow-through (lane 2) and little protein on the chitin resin (lane 3). The W687F/W696F double mutant showed essentially the same binding efficiency as the W687F mutant, suggesting that there was no cumulative effect between those two residues. Finally, substitution of Glu688, a conserved charged residue on the surface, with alanine or glutamine did not significantly affect the binding to chitin at both low and high salt concentrations (Figure 3A). Thus, these conserved residues do not appear to be essential for chitin binding.

EXAMPLE V

EFFECT OF IONIC STRENGTH ON CHITIN-BINDING AND ELUTION

To further analyze the effect of ionic strength on the affinity to chitin, binding of the W687F mutant was assessed in buffer containing 2 M, 1 M, 0.5 M or 50 mM NaCl. As shown in Figure 4A, the binding efficiency of the W687F mutant

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correlated with the ionic strength of the buffer. Increasing the NaCl concentration from 50 mM to 0.5 M or 1 M resulted in partial binding of PXB (W687F) protein, as indicated by the presence of PXB in a sample of chitin resin after loading and washing (0.5 M and 1 M, lane 3). In contrast, binding at 2 M NaCl concentration permitted efficient binding to chitin indicated by the presence of only a trace amount of the PXB species in the chitin flow-through (2 M, lane 2) and the presence of PXB in the chitin resin fraction (2 M, lane 3).

The observation that the W687F mutant protein was incapable of binding chitin in 50 mM NaCl implied that the elution of the bound CBD fusion protein might be conducted by lowering NaCl concentration. Indeed, after loading and washing at 2 M NaCl, PXB proteins were efficiently eluted in buffer containing 50 mM or no NaCl (lanes 4 and 5, Figure 4A). We further examined whether the chitin binding activity of the W687F mutant is reversible by adjusting the NaCl concentration of the eluted protein from 50 mM to 2 M NaCl. PXB fusion protein bound to chitin efficiently since it was completely depleted in the chitin flow-through and was present in the chitin resin fraction (lanes 6 and 7, Figure 4A). Furthermore, different ionic strengths were used for elution after absorption and wash in the buffer containing 2 M NaCl (Figure 4B). In the control experiment, the PXB protein possessing the wild type CBD exhibited a very weak elution with buffer containing 50 mM NaCl. The assay of the W687F mutant showed that 50 mM NaCl was sufficient for release of

the mutant protein from chitin. Although the protein was partially eluted in buffers containing 0.1 to 1 M NaCl less protein was released from chitin beads in correlation to the increase in ionic strength.

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EXAMPLE VI

ONE-STEP AFFINITY PURIFICATION OF CBD FUSION PROTEINS

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Modification of CBD for example using the CBD (W687F) mutant resulted in an elutable affinity tag for single column purification of recombinant proteins. A protein fused to the mutated CBD could be purified by chitin resin in a high salt buffer (e.g. 2 M NaCl) and released by simply shifting the NaCl concentration in the elution buffer to 50 mM NaCl (Figure 5A). This was further demonstrated using human granulocyte-macrophage colony stimulating factor (hGM-CSF) and the kinase domain of Her-2 [Her-2 (KD)] both fused at their C-terminus to the mutated chitin binding domain (Figure 5B and Figure 5C).

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Expression of pGM-CSF-CBD or pHer-2(KD)-CBD in *E. coli* ER2566 cells was carried out at 30°C for 3 hours in the presence of 0.3 mM IPTG when cell-density reached an A_{600} of 0.5-0.7. Induced cells were collected by centrifugation and resuspended in 20 mM Tris-HCl (pH 8) containing 0.5 M NaCl. Both fusion proteins were found in inclusion bodies which were isolated by breaking cells by sonication followed by centrifugation at $15,000 \times g$ for 30 min. The proteins were

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then solubilized in 20 mM Tris-HCl (pH 8) containing 0.5 M NaCl, 7 M Guanidine-HCl and 10 mM DTT and insoluble components were removed by centrifugation at $15,000 \times g$ for 30 min. To renature insoluble fusion proteins, the supernatant was dialyzed successively at 4°C against 20 mM Tris-HCl (pH 8) and 0.5 M NaCl containing: 8 M urea, 10 mM DTT; 6 M urea, 1 mM DTT; 4 M urea, 1 mM DTT; 2 M urea, 0.1 mM oxidized glutathione, 1 mM reduced glutathione. Renatured fusion proteins were dialyzed twice against 20 mM Tris-HCl, 0.5 M NaCl containing 0.1 mM oxidized glutathione, 1 mM reduced glutathione, and no urea. Insoluble components were removed by centrifugation at $15,000 \times g$ for 30 min and the final NaCl concentration was increased to 2 M. Binding was performed by loading the supernatant onto a 25 ml chitin resin equilibrated in 20 mM Tris-HCl (pH 8) containing 2 M NaCl. The column was washed with 30 column volumes of the same buffer and then flushed with the elution buffer containing 20 mM Tris-HCl (pH 8) and 50 mM NaCl. Proteins were analyzed with a 12% Tris-glycine gel and the concentration was determined by Bradford assay.

Both hGM-CSF-CBD and Her-2(KD)-CBD fusion proteins were expressed in *E. coli* strain ER 2566 as inclusion bodies and consequently absent in the clarified cell extract after centrifugation (Figure 5, lane 3). After solubilization and renaturation steps, these fusion proteins remained soluble and were applied to chitin resin at 2 M NaCl. Analysis of chitin beads after loading and washing at 2 M confirmed that both

recombinant proteins were absorbed onto chitin (lane 6). Binding was approximately 80% for hGM-CSF-CBD and essentially 100% for Her-2(KD)-CBD (lane 5). Analysis by SDS-PAGE showed a prominent and single band corresponding to the expected hGM-CSF-CBD and Her-2(KD)-CBD fusion protein when a buffer containing 50 mM NaCl was used to elute the bound fusion proteins (lane 7). The obtained yields were 12.7 mg fusion protein per 1 liter cell culture for hGM-CSF-CBD and 4.5 mg per liter for Her-2(KD)-CBD.

EXAMPLE VII

METHOD FOR SECRETING AND PURIFYING BOVINE ENTEROKINASE FROM *KLUYVEROMYCES LACTIS*

A DNA fragment encoding bovine enterokinase with a c-terminal mutant CBD fusion [EK-CBD(W275F)] was created by PCR amplification from a template consisting of a *K. lactis* expression vector containing enterokinase with a wild-type CBD fusion (pEK-CBD). The forward primer for amplification was 5'-CCGCTCGAGAAAAGAATTGTTGGTGGTTCTGATTCTAGA-3' (SEQ ID NO:40) and the reverse primer 5'-ATAAGAATGCGGC CGCTCATTGAAGCTGCCACAAGGCAGGAACGTTGGATGGTTCA**AA**ATC CTGCC-3' (SEQ ID NO:41). The reverse primer directs incorporation of a 2 bp mutation into the CBD region (bold/underline) thus converting it from wild-type to the W275F mutant form. To facilitate subcloning, forward and reverse primers also contained sequences for *Xho*I and *Not*I restrictions sites (single underline), respectively. PCR conditions consisted of Vent® DNA polymerase in 20 mM Tris-

-40-

HCl (pH 8.8 at 25°C) containing 10 mM KCl, 10 mM (NH₄)₂SO₄, 4mM MgSO₄, and 0.1% Triton X-100. The reaction mixture (50 µl total volume) contained 1 µM of each primer, 200 µM dNTPs and 60 ng of pEK-CBD. The reaction was initiated using a
5 "hot start" procedure consisting of incubation at 95°C for 5 min then 80°C for 1 min at which time 2 U Vent® DNA polymerase was added. Thermocycling was performed for 30 cycles of successive incubations at 95°C for 30 s, 59°C for 30 s and 72°C for 1 min, followed by a final 72°C incubation for 5
10 min. The PCR product was purified via the QIAQuick PCR Isolation Kit (Qiagen Inc., Studio City, CA).

All of the purified DNA was cleaved with *Not*I and *Xho*I as follows: purified PCR product (30µl) was mixed with 5 µl of
15 10X NEBuffer 3 (500 mM Tris-HCl, 100 mM MgCl₂, 1 M NaCl, 10 mM dithiothreitol), 1 µl BSA (10 mg/ml), 1 µl each of both *Not*I (20 U) and *Xho*I (20 U), and distilled water (12 µl), followed by incubation at 37°C for 2 h. The reaction was terminated by heating to 65°C for 10 min, and the cleaved DNA purified via
20 the QIAQuick PCR Isolation Kit. The cleaved DNA was ligated into *Not*I-*Xho*I cleaved pGBN2 (an integration vector for *K. lactis* expression) as follows: 500 ng of *Not*I-*Xho*I cleaved PCR product (3 µl) was mixed with 500 ng of *Not*I-*Xho*I cleaved pGBN2 (3 µl), 2 µl of 10X Ligation Buffer (500 mM Tris pH 7.5, 100 mM MgCl₂, 100 mM dithiothreitol, 5 mM ATP), 11 µl distilled
25 water, and 1µl (1 U) of ligase, and incubated for 15 h at 4°C. The ligation was desalted by microdialysis on a 0.025 µm membrane (Millipore Inc., Bedford, MA) at RT for 30 min.

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Ligated DNA (10 μ l) was electroporated into *E. coli* ER2268. *E. coli* was prepared for electroporation by growing 1 L of cells to an optical density of 0.5 in L-broth. The cells were chilled on ice for 15 to 30 min then pelleted at 4°C by centrifugation at 4000 rpm for 10 min. The cell pellet was washed twice in sterile cold water and once in cold 10% glycerol, then resuspended in 2 ml 10% glycerol to a final cell concentration of $\sim 3 \times 10^{10}$ cells per ml. Cells were stored in 70 μ l aliquots at -70°C until needed. To electroporate DNA into *E. coli*, frozen cells were thawed on ice and mixed with 10 μ l of the microdialyzed ligation. The mixture was placed into a cold 0.2 cm electroporation cuvette and a pulse of electricity (2.5 KV, 25 μ F and 200 Ohm) was applied to the cell mixture. The *E. coli* was immediately diluted with 1 ml L-broth, grown at 37°C for 30 min, and plated on L-agar plates containing 100 μ g/ml ampicillin. After overnight incubation, screening for colonies carrying plasmids that successfully ligated the PCR product (described above) was performed by growing 10 ml cultures (L-broth with 100 μ g/ml ampicillin) from 10 single transformants. Plasmid DNA was isolated from each culture using the QIAprep Spin Miniprep Kit from Qiagen Inc. (Studio City, CA). Clones with inserts were identified by digesting miniprep DNA as follows: 5 μ l (1 μ g) plasmid DNA was mixed with 5 μ l of 10X NEBuffer 3 (500 mM Tris-HCl, 100 mM MgCl₂, 1 M NaCl, 10 mM dithiothreitol), 1 μ l BSA (10 mg/ml), 1 μ l each of both *Not*I (20 U) and *Xho*I (20 U), and 38 μ l deionized water, followed by incubation at 37°C for 2 h. Digested DNAs were subjected to electrophoresis through a 1% agarose gel in

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Tris-Acetate-EDTA (TAE) buffer. Clones containing a 903 bp insert were subjected to automated sequencing using pGBN2-specific primers 5'-TCCGAGCTCAAAACAATGAGATTTCTTCAAT TTTTACT-3' (forward) (SEQ ID NO:42) and 5'-GCATGTATACAT CAGTATCTC-3' (reverse) (SEQ ID NO:43) to confirm proper incorporation of the 2 bp mutation into the CBD region.

EXAMPLE VIII

SECRETED EXPRESSION OF EK-CBD(W275F) IN *K. LACTIS*

To integrate DNA encoding EK-CBD(W275F) into the chromosome of *K. lactis* for expression, clones of DNA encoding EK-CBD(W275F) in pGBN2 were first linearized by digestion with *Sac*II as follows: 15 µl (3 µg) plasmid DNA, 5 µl 10 X NEBuffer 4 (200 mM Tris-acetate, 100 mM magnesium acetate, 500 mM potassium acetate, 10 mM dithiothreitol), 2 µl (40 U) *Sac*II , and 28 µl deionized water were mixed and incubated at 37°C for 4h. Digested vector was purified via the QIAQuick PCR Isolation Kit (Qiagen Inc., Studio City, CA) and eluted in 30 µl deionized water. Linearized DNA (10 µl) was introduced into *K. lactis* GG799 and integrated into the genome at the *LAC4* locus. *K. lactis* was prepared for electroporation by growing 100 ml of cells to an optical density of 1.0 in YPD broth (10 g yeast extract, 20 g peptone and 1% dextrose per liter). The cells were chilled on ice for 10 min then pelleted at 4°C by centrifugation at 4000 rpm for 10 min. The pellet was washed with 100 ml sterile cold water and with 5 ml sterile cold 1 M sorbitol, then resuspended in

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0.1 ml sterile cold 1 M sorbitol to a final volume of ~0.3 ml. The cells were stored on ice until use. To electroporate DNA into the prepared cells, 70 μ l of *K. lactis* cell suspension was mixed with 10 μ l of purified *Sac*II digested expression vector. The mixture was placed into a cold 0.2 cm electroporation cuvette and a pulse of electricity (1.5 KV, 25 μ F and 200 Ohm) was applied to the cell mixture. The cells were immediately diluted with 1 ml sterile cold 1 M sorbitol and placed on ice for 10 min after which 1 ml of YPD was added and the cells grown at 30°C for 2h. Cells were plated on YPD agar plates containing 200 μ g/ml G418 and colonies of integrants allowed to form by incubation at 30°C for 3 days. Secretion of EK-CBD(W275F) was achieved by growing integrants in YPD broth for 24-96 h. Enterokinase proteolytic activity associated with secreted EK-CBD(W275F) was assayed directly from culture medium in a fluorogenic assay as follows: 50 μ l of culture supernatant was mixed with 50 μ l of 2X assay buffer (875 μ M fluorescent peptide (H-Gly-Asp-Asp-Asp-Asp-Lys- β NA (SEQ ID NO:44)), 17.6% DMSO, 125 mM Tris pH 8.0) and incubated at RT for 5-30 min. Released fluorescence compared to a standard reaction (50 μ l YPD and 50 μ l 2X assay buffer) was measured with a Perkin Elmer (Emeryville, CA) LS50B Luminescence Spectrophotometer with excitation and emission wavelengths of 337 nm and 420 nm, respectively.

EXAMPLE IX
CHITIN BEAD AFFINITY PURIFICATION OF
EK-CBD(W275F) ACTIVITY

5 EK-CBD(W275F) was purified directly from culture media
using a batch method as follows: a 250 ml YPD-broth culture
of a *K. lactis* EK-CBD(W275F) secreting strain was grown at
30°C for 48 h. The culture was cleared of cells by
centrifugation at 4000 rpm for 10 min at 4°C. Cleared culture
10 was adjusted to 2 M NaCl by addition of 29.22 g of solid NaCl
to promote binding of EK-CBD(W275F) to chitin beads. New
England Biolabs (Beverly, MA) chitin bead suspension (5 ml)
was added to the cleared culture and the beads were gently
stirred for 2 hours at 4°C. The entire mixture was passed
15 through an empty 23 cm x 2.3 cm column to collect the EK-
CBD(W275F)-bound chitin beads. Collected beads were
washed by passing 75 ml of wash buffer (2 M NaCl, 20 mM
Tris pH 7.4) through the column. EK-CBD(W275F) was eluted
from the chitin beads by passing 25 ml of elution buffer (50
20 mM NaCl, 20 mM Tris pH 7.4) through the column while
collecting 0.5 ml fractions. Fractions were assayed for
enterokinase activity as follows: 50 µl of a fraction was mixed
with 50 µl of 2X assay buffer (875 µM fluorescent peptide (H-
Gly-Asp-Asp-Asp-Asp-Lys-βNA) (SEQ ID NO:45), 17.6% DMSO,
25 125 mM Tris pH 8.0) and incubated at RT for 5-30 min.
Released fluorescence compared to a standard reaction (50 µl
elution buffer and 50 µl 2X assay buffer) was measured with
a Perkin Elmer (Emeryville, CA) LS50B Luminescence

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Spectrophotometer with excitation and emission wavelengths of 337 nm and 420 nm, respectively. Fractions having activity were pooled and stored frozen at -20°C .

5 Although certain preferred embodiments of the present invention have been described, the spirit and scope of the invention is by no means restricted to what is described above. For example, within the general method for secreting CBD-tagged enterokinase, it is also possible to express and
10 purify other CBD-tagged proteins from both *K. lactis* as well as from other yeast and fungi, or from other eukaryotic or prokaryotic secretory or cytosolic expression systems.

WHAT IS CLAIMED IS:

1. A protein comprising: a chitin binding domain (CBD)
capable of reversibly binding a chitin substrate under a
selected non-denaturing condition.
2. A protein according to claim 1, wherein the CBD has one
or more mutated amino acids.
3. A protein according to claim 2, wherein the mutated
amino acid is an aromatic amino acid.
4. A protein according to claim 2, wherein the mutated
amino acid is positioned within a binding cleft of the
CBD.
5. A protein according to claim 3, wherein the amino acid
comprises a tryptophan.
6. A protein according to claim 5, wherein the tryptophan
corresponds to tryptophan 687 of *B. circulans* chitinase
A1.
7. A protein according to claim 1, wherein the non-
denaturing conditions are selected from changes in any
of: ionic conditions; pH; detergent concentration, or
antagonist or agonist concentration.

8. A protein according to claim 1, wherein the non-denaturing conditions comprise a change in ionic conditions.

5 9. A protein according to claim 8, wherein the change in ionic conditions comprise a reduced salt concentration.

10 10. A method of obtaining a chitin binding domain (CBD) capable of reversibly binding to a chitin substrate under non-denaturing conditions, the method comprising:
(a) modifying at least one amino acid within the CBD;
(b) selecting the modified CBD according to its binding to the chitin substrate; and
(c) determining whether the modified CBD is capable
15 of reversibly binding chitin under altered conditions.

11. A method according to claim 10, wherein modifying at least one amino acid within the CBD, further comprises:
mutating a portion of a DNA sequence encoding
20 the CBD and expressing the DNA in a host cell or by *in vitro* translation.

12. A method according to claim 11, wherein mutating a portion of the DNA sequence, further comprises:
25 substituting with an oligonucleotide, a portion of the DNA sequence encoding the CBD, the oligonucleotide differing from the substituted portion by a mutation at a target site.

13. A method according to claim 12, wherein the target site is a tryptophan and the mutation is a phenylalanine substitution of the tryptophan.

5

14. A method according to claim 10, wherein the selected condition comprises altering ionic conditions.

10

15. A method according to claim 10, wherein the selected condition comprises changing NaCl molarity.

16. A method for producing and purifying a target protein molecule comprising:

15

(a) constructing a DNA expression vector which expresses a hybrid polypeptide in a transformed host cell, the hybrid polypeptide comprising the target protein molecule and a modified chitin binding domain having a specific and reversible affinity for a substrate which binds to the chitin binding domain;

20

(b) introducing the expression vector into an appropriate host cell and expressing the hybrid polypeptide;

25

(c) contacting the hybrid polypeptide produced by the transformed cell with the substrate to which the CBD binds; and

(d) recovering the hybrid polypeptide.

17. A method according to claim 16, wherein recovering the hybrid polypeptide comprises altering the ionic conditions.

5 18. The method of claim 16, wherein recovery of the hybrid polypeptide further comprises:

contacting the bound hybrid polypeptide with a substance which displaces the hybrid polypeptide.

10 19. A method according to claim 17, wherein the altered ionic condition is a reduction in NaCl molarity.

20. A method according to claim 16, wherein the substrate is chitin.

15 21. A method for purifying a chitin binding domain-target molecule conjugate from a mixture of molecules in a preparation comprising:

(a) adding to the preparation, a substrate having a specific and reversible affinity for CBD so as to permit binding and immobilizing of the conjugate to the substrate;

(b) removing the bound conjugate from the mixture into a buffer; and

20 25 (c) altering the ionic conditions in the buffer so as to elute the conjugate from the substrate into the buffer to obtain the purified conjugate.

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22. A method according to claim 21, wherein the altered ionic conditions comprise a change in NaCl molarity.
23. A method according to claim 21, wherein the substrate
5 is chitin.
24. A kit for purifying a recombinant protein, comprising:
(a) a plasmid, the plasmid containing a DNA sequence
encoding a mutant CBD which is capable of reversibly
10 binding a chitin matrix and an insertion site for inserting
the DNA sequence encoding the recombinant protein;
and
(b) the chitin matrix for binding the fusion protein; and
optionally a buffer for eluting the fusion protein from the
15 chitin matrix.
25. A fusion vector for constructing an expression vector
which expresses a modified chitin binding domain (CBD)
fused to a protein molecule to be purified, comprising:
20 a DNA fragment coding for the modified chitin
binding domain or portion thereof, having a specific and
reversible affinity for a substrate which binds to the
chitin binding protein.
26. A DNA expression vector for producing a purified protein
25 molecule, which upon expression produces a chitin
binding domain fused to the protein molecule,
comprising:

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(a) a first DNA fragment coding for the chitin binding domain (CBD) or portion thereof having a specific and reversible affinity for substrate which binds to the chitin binding protein; and

(b) a second DNA fragment coding for the protein molecule to be purified.

27. A method according to claims 25 or 26 wherein the substrate is chitin.

28. A vector comprising a sequence for a mutant CBD and optionally an insertion site for placing a sequence encoding a recombinant protein within or adjacent to the CBD sequence.

29. A vector according to claim 28, wherein the mutation is at Try 687

30. A host cell for making a fusion protein, comprising: a vector according to claims 25, 26 or 28.

FIG. 1

B. circulans ChiA1 (SEQ ID NO:1)
 Aeromonas ChiII (SEQ ID NO:3)
 S. marcescens ChiC (SEQ ID NO:5)
 Aeromonas ORF1 (SEQ ID NO:7)
 S. marcescens ChiB (SEQ ID NO:9)
 Alteromonas Chi85 (SEQ ID NO:11)
 V. harveyi ChiA (SEQ ID NO:13)

B. circulans ChiD (SEQ ID NO:2)
 J. lividum Chitinase (SEQ ID NO:4)
 Aeromonas ChiII (SEQ ID NO:6)
 Aeromonas ChiI (SEQ ID NO:8)
 J. lividum Chitinase (SEQ ID NO:10)
 S. griseus ChiC (SEQ ID NO:12)
 A. cavia ChiA (SEQ ID NO:14)

B. circulans ChiA1	660	670	680	690	699
B. circulans ChiD					77
Aeromonas ChiII					542
J. lividum Chitinase					106
S. marcescens ChiC					480
Aeromonas ChiII					168
Aeromonas ORF1					438
Aeromonas ChiI					499
S. marcescens ChiB					200
J. lividum Chitinase86					820
Alteromonas Chi85					81
S. griseus ChiC					557
V. harveyi ChiA					865
A. cavia ChiA					

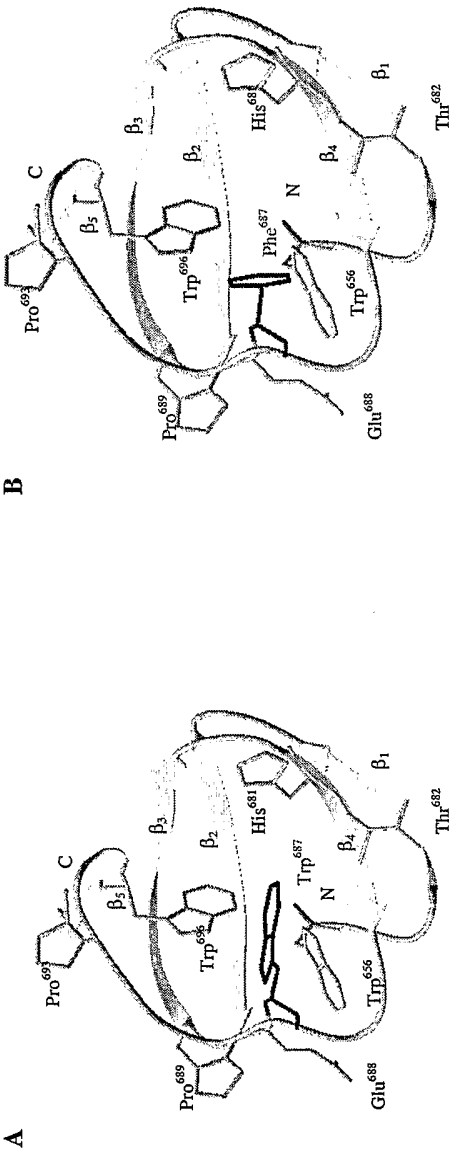


Figure 2

FIG. 3A

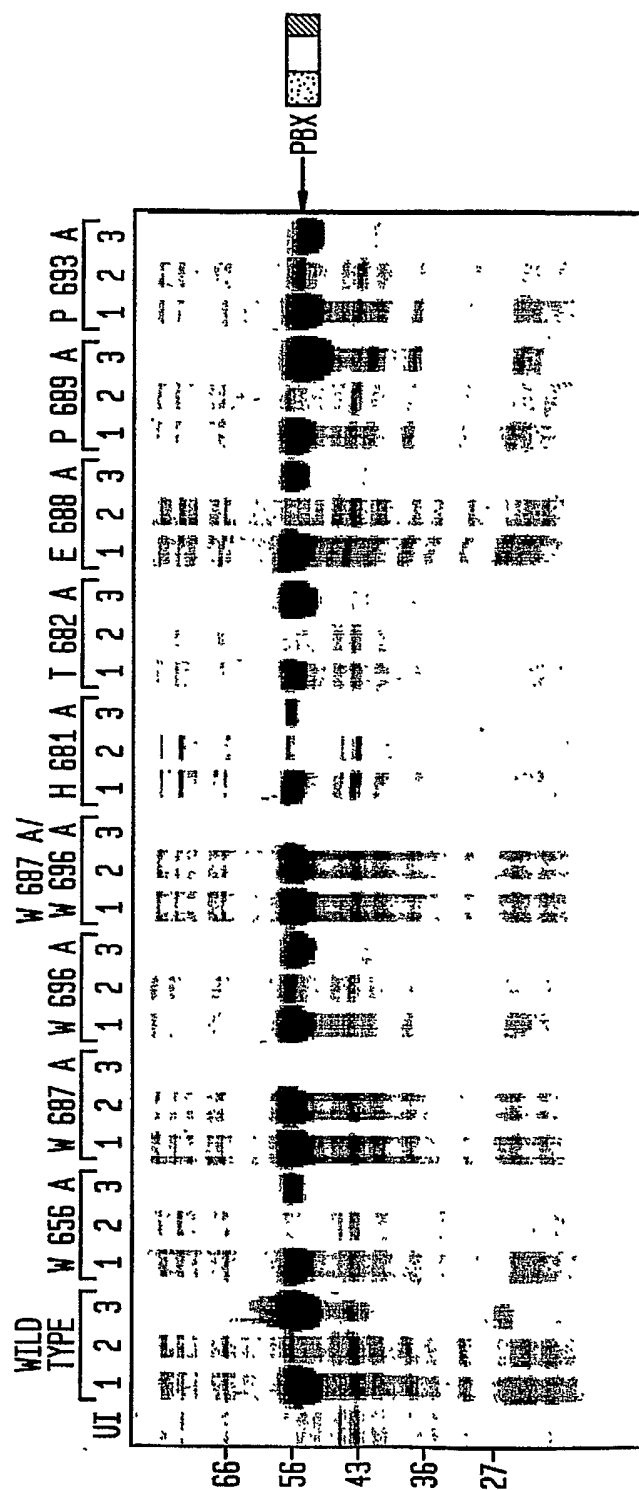


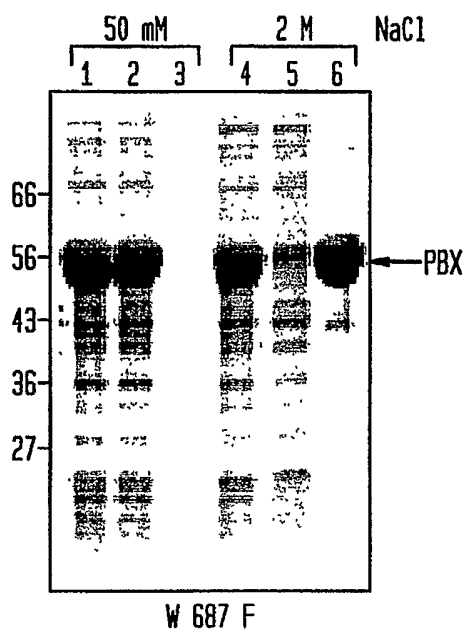
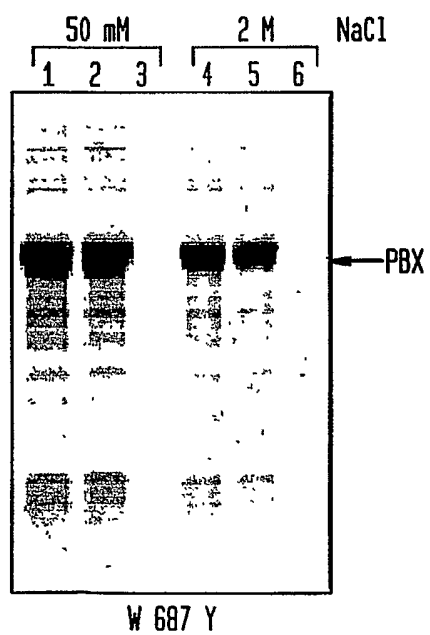
FIG. 3B**FIG. 3C**

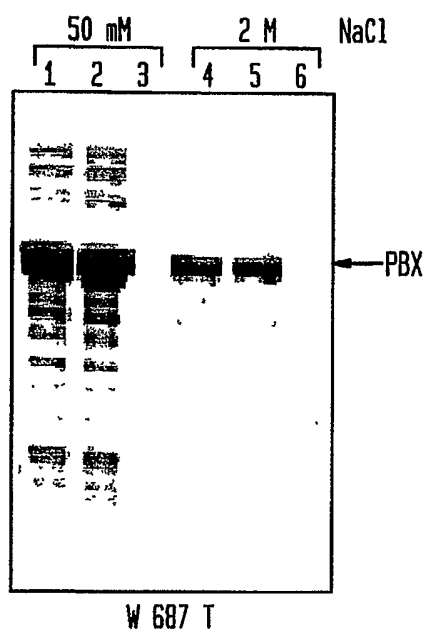
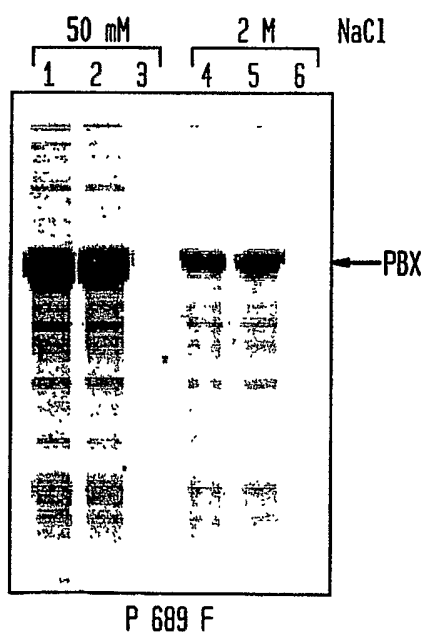
FIG. 3D**FIG. 3E**

FIG. 4A

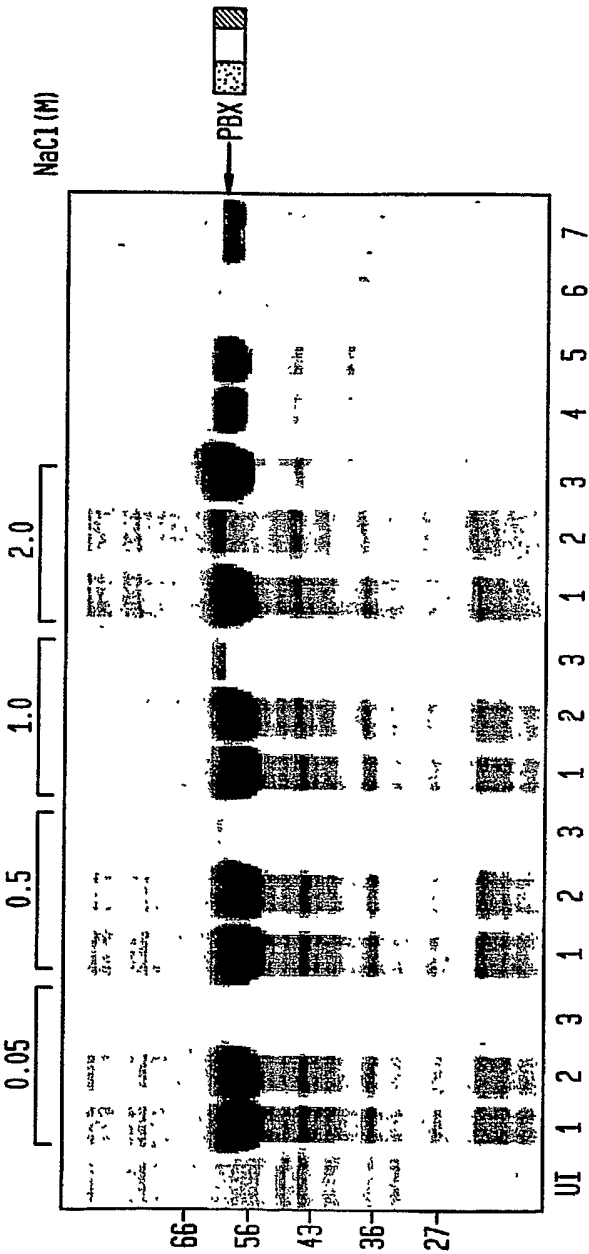


FIG. 4B

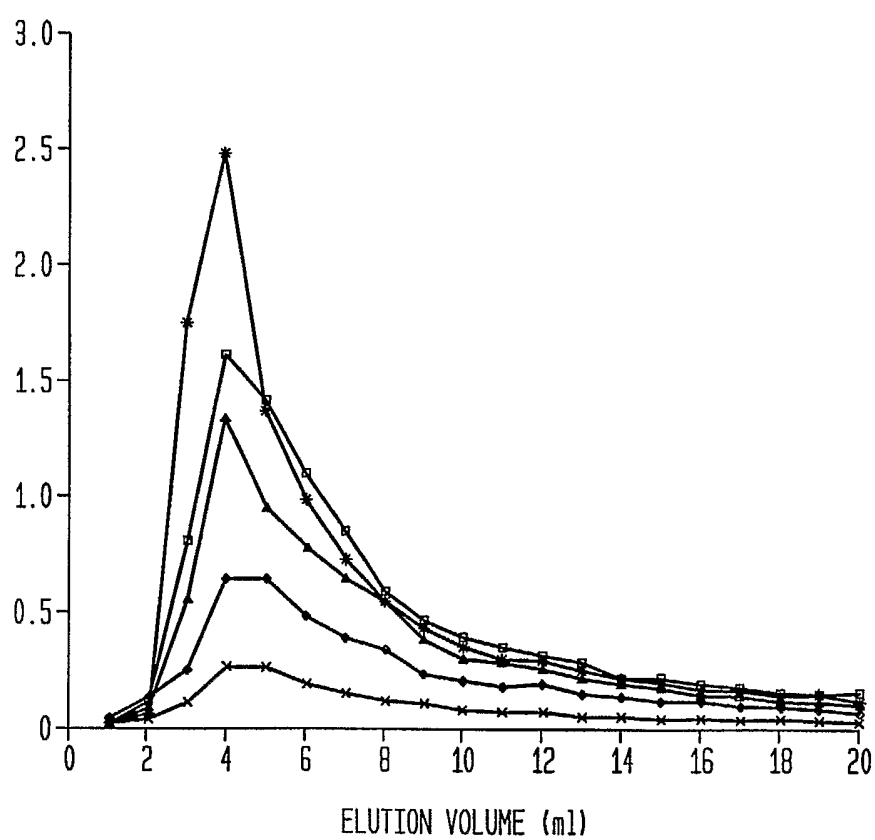


FIG. 5A

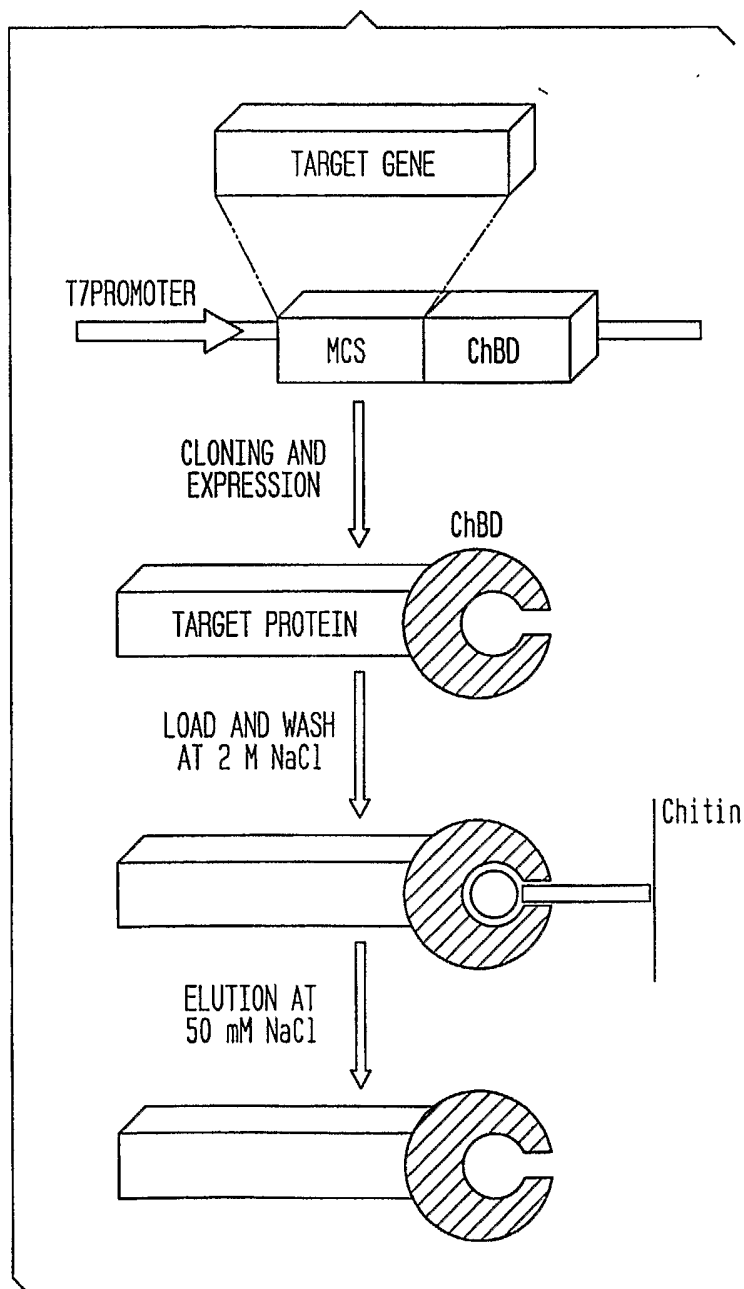
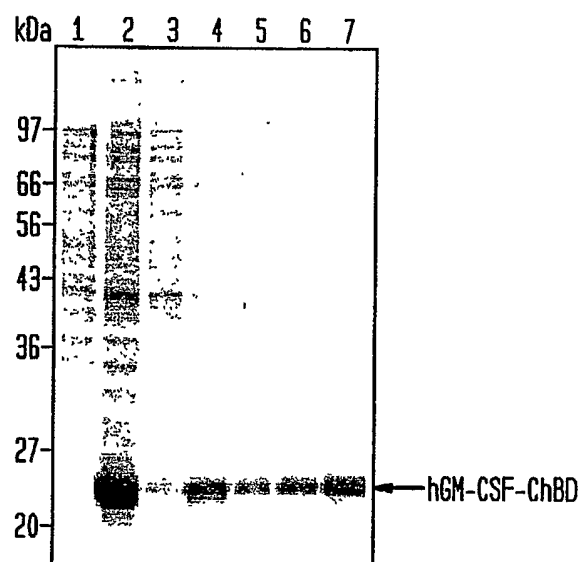
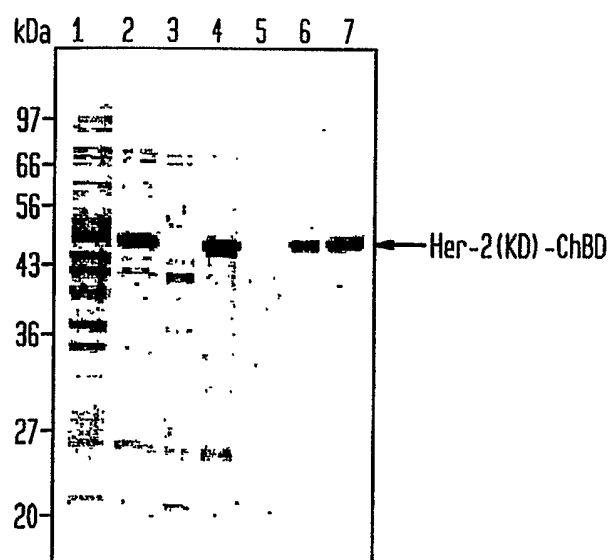


FIG. 5B**FIG. 5C**

SEQUENCE LISTING

<110> New England Biolabs, Inc.
 Xu, Ming-Qun
 Ferrandon, Sebastien
 Taron, Christopher
 Colussi, Paul

<120> Modified Chitin Binding Domain And Uses Thereof

<130> NEB-200-PCT

<150> US 60/360,354

<151> 2002-02-28

<160> 49

<170> PatentIn version 3.1

<210> 1

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<212> PRT

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Ala	Trp	Gln	Val	Asn	Thr	Ala	Tyr	Thr	Ala	Gly	Gln	Leu	Val	Thr	Tyr
1				5					10					15	

Asn	Gly	Lys	Thr	Tyr	Lys	Cys	Leu	Gln	Pro	His	Thr	Ser	Leu	Ala	Gly
			20					25					30		

Trp	Glu	Pro	Ser	Asn	Val	Pro	Ala	Leu	Trp	Gln	Leu	Gln
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<212> PRT

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			20					25					30		

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			35				40					45		

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 1 5 10 15

Thr Cys Ala Ser Tyr Gly Gly Lys Asp Tyr Val Ala Gln Val Thr His
 20 25 30

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 50

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 1 5 10 15

Thr Val Thr Tyr Leu Gly Gly Asn Tyr Thr Ala Leu Val Thr Gln Thr
 20 25 30

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Asp Val Val Ser Tyr Lys Gly Lys Lys Tyr Thr Cys Ile Gln Ala His
 20 25 30

Thr Ser Asn Ala Gly Trp Thr Pro Asp Ala Ala Phe Thr Leu Trp Gln
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Leu Ile Ala
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Ser Tyr Asn Gly His Thr Tyr Thr Ala Lys Trp Trp Thr Gln Gly Asn
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Val Pro Ser Ser Ser Thr Gly Asp Gly Ser Pro Trp Asn Asp Val
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 20 25 30

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30