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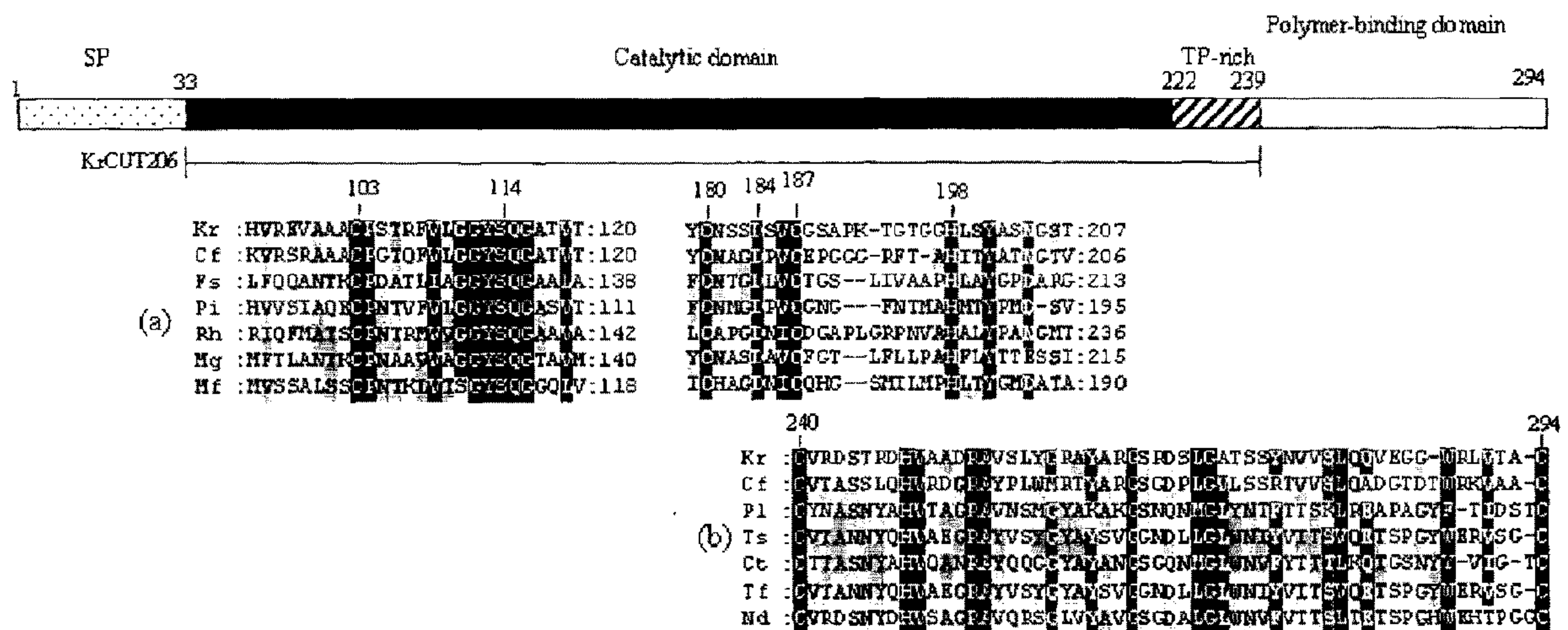


Figure 1

(57) Abrégé/Abstract:

Multi-domain recombinant proteins having a cutinase catalytic domain are provided. The cutinase catalytic domain is a modular domain, which may be combined with other protein domains that also function in a modular fashion. For example, the cutinase catalytic domain can be operably linked to a polymer binding domain via a linker domain (e.g., a threonine/proline-rich linker polypeptide). In some embodiments, the cutinase catalytic domain is from an endogenous cutinase having a multi-domain modular organization, such as from the actinobacterium *Kineococcus radiotolerans*. Compositions, methods and uses of the recombinant proteins are also disclosed, such as for use in industrial applications.

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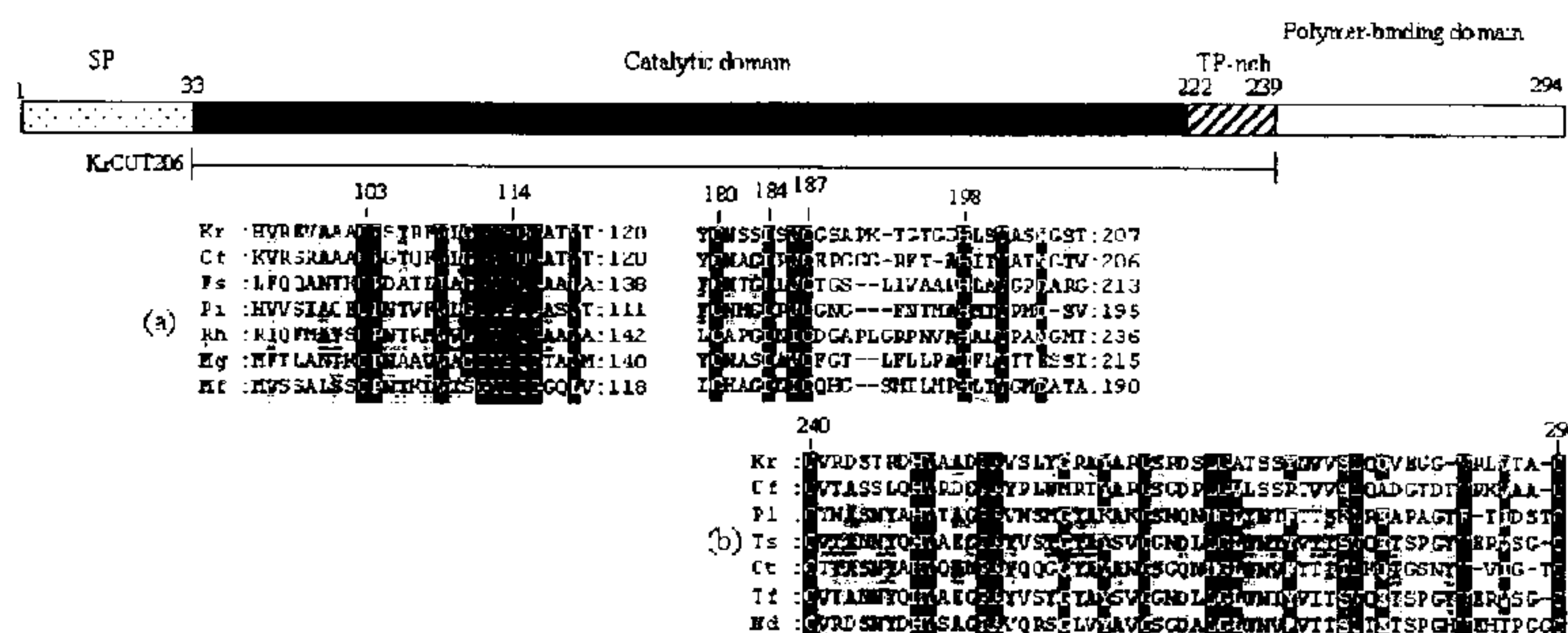


Figure 1

(57) Abstract: Multi-domain recombinant proteins having a cutinase catalytic domain are provided. The cutinase catalytic domain is a modular domain, which may be combined with other protein domains that also function in a modular fashion. For example, the cutinase catalytic domain can be operably linked to a polymer binding domain via a linker domain (e.g., a threonine/proline-rich linker polypeptide). In some embodiments, the cutinase catalytic domain is from an endogenous cutinase having a multi-domain modular organization, such as from the actinobacterium Kineococcus radiotolerans. Compositions, methods and uses of the recombinant proteins are also disclosed, such as for use in industrial applications.

MULTI-DOMAIN ENZYMES HAVING CUTINASE ACTIVITY, COMPOSITIONS COMPRISING SAME AND USES THEREOF

FIELD

5 The present invention relates to enzymes with cutinase activity and methods for transgenic expression thereof, and various uses thereof. More specifically, the present invention is concerned with enzymes with cutinase activity having a modular organization, methods relating to their transgenic expression, compositions comprising same, and uses thereof.

10 BACKGROUND

 Cutinases (EC 3.1.1.74) are hydrolytic enzymes, i.e. hydrolases, that degrade cutin, a component of the plant cuticle that, in addition to waxes, constitute the outermost continuous membrane or "skin" of the primary parts of higher plants. Cutin is a polyester, largely composed of saturated C₁₆ (palmitic) acids and unsaturated C₁₈ fatty acids cross-linked by ester bonds; the actual composition of which depends on the plant species. The cuticle primarily
15 protects plants from water loss or other environmental stresses, thereby acting as a formidable barrier.

 Cutinases have been found predominantly in phytopathogenic fungi as secreted enzymes to facilitate their entry into the host plant. The prototypical cutinase is the FsCUT derived from *Fusarium solani* f. sp. *pisi* (aka *Nectria haematococca*) that infects peas. This enzyme plays a role in plant pathogenesis as a virulence factor, but not exclusively since hydrophobic surface binding proteins, called hydrophobins, are also involved. FsCUT is a 230-
20 amino acid enzyme synthesized with a 31 amino-acid signal peptide, with the mature, secreted portion having a molecular mass of 21,600 Da (Soliday et al., 1984). Its structure belongs to the α/β class of hydrolases containing the classical Ser-His-Asp triad (S120, H188 and D175) for catalysis (Martinez et al., 1992).

 Owing to the hydrolytic, esterification or transesterification activities, both naturally-occurring or genetically-modified cutinases of *Fusarium* and other organisms, have been produced and applied to varying extent in industries
25 such as food, laundry and detergent, textiles, recycling, and polymer manufacturing.

SUMMARY

 The present description stems from the surprising discovery that members of a family of multi-domain enzymes having cutinase activity may have advantageous properties for various industrial applications. In some implementations, these advantageous properties may include improved stability in the presence of an oxidizing
30 agent, improved stability in the presence of an organic solvent, and/or improved enzymatic activity at a broader range of temperatures, relative to prototypical single-domain cutinases.

 This family of multi-domain enzymes having cutinase activity includes an endogenous enzyme (KrCUT) from the actinobacterium *Kineococcus radiotolerans*, a highly radiation-resistant actinobacterium isolated from nuclear waste at the Savannah River Site in Aiken, South Carolina, as well as enzymes from *Cellulomonas*

bogoriensis, *Cellulomonas cellasea*, and *Cellulomonas flavigena*. This family of enzymes differs from prototypical cutinases such as those from the filamentous fungus *Fusarium solani*, in that they have a multi-domain modular organization in the form of a cutinase catalytic domain that has an extra C-terminal segment containing a threonine/proline-rich (T/P-rich) linker domain, and polymer binding domain. Recombinant proteins having cutinase activity having different improved properties and substrate preferences can thus be engineered using cutinase catalytic domains, linker domains, and polymer binding domains present in cutinases from different organisms.

In some implementations, the present description relates to one or more of the following items.

1. An isolated recombinant protein having cutinase activity, said protein comprising: (a) a cutinase catalytic domain; (b) a proline/threonine-rich linker domain positioned C-terminal of domain (a); and (c) a polymer binding domain positioned C-terminal of domain (b).

2. The isolated recombinant protein of item 1, wherein said protein, as compared to a corresponding native protein comprising domain (a):

(i) has improved stability to a bleaching agent which is:

(1) an oxidizing agent which is at least one of: hydrogen peroxide, a peroxide other than hydrogen peroxide, a non-peroxide oxidizing agent, ozone, sodium percarbonate, sodium perborate, or any combination thereof; and/or

(2) a reducing agent which is at least one of: sodium dithionite, sodium dithionite, sulfur dioxide, a sulfite, a bisulfite, a sodium borohydride, or any combination thereof;

(ii) has improved stability to an organic solvent which is at least one of: methanol, 2-propanol, or another alcohol, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), acetone, or another organic solvent, or any combination thereof;

(iii) has a broader temperature activity range; or

(iv) any combination of (i) to (iii).

3. The isolated recombinant protein of item 1 or 2 which is a chimeric recombinant protein, wherein at least one of domains (a), (b) and (c) is from a cutinase belonging to a different species.

4. The isolated recombinant protein of any one of items 1 to 3, wherein domain (a), (b), and/or (c) is from a multi-domain cutinase belonging to *Kineococcus radiotolerans*, *Cellulomonas flavigena*, *Cellulomonas bogoriensis*, or *Cellulomonas cellasea*.

5. The isolated recombinant protein of any one of claims 1 to 4, wherein:

(i) domain (a) is:

- (1) a cutinase catalytic domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to amino acid sequence of SEQ ID NOs: 2, 30, 33, 36, 39, or 42-45, or to the cutinase catalytic domain of SEQ ID NO: 1, 13-20, or 25;
- 5 (2) a cutinase catalytic domain comprising or consisting of the amino acid sequence of SEQ ID NOs: 2, 30, 33, 36, 39, or 42-45, or the cutinase catalytic domain of SEQ ID NO: 1, 13-20, or 25; or
- (3) both (1) and (2);
- (ii) domain (b) is:
- 10 (1) a threonine/proline-rich linker domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to SEQ ID NO: 3, 31, 34, 37, or 40, or to the threonine/proline-rich linker domain of SEQ ID NO: 1 or 13-15;
- (2) a threonine/proline-rich linker domain comprising or consisting of the amino acid sequence of SEQ ID NO: 3, 31, 34, 37, or 40, or to the threonine/proline-rich linker domain of SEQ ID NO: 1 or 13-15;
- 15 (3) a threonine/proline-rich linker domain having at threonine/proline content of at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% over a stretch of 15 to 55 consecutive amino acids; or
- 20 (4) any combination of (1) to (3);
- (iii) domain (c) is:
- (1) a polymer binding domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to SEQ ID NO: 4, 32, 35, 38, or 41;
- 25 (2) a polymer binding domain comprising or consisting of the amino acid sequence of SEQ ID NO: 4, 32, 35, 38, or 41, or to the polymer binding domain of SEQ ID NO: 1 or 13-15; or
- (3) both (1) and (2); or
- (iv) any combination of (i) to (iii).
- 30 6. The isolated recombinant protein of any one of items 1 to 5, wherein said recombinant protein:
- (i) is produced fused to a heterologous carrier protein or heterologous tag that enables secretion of said recombinant protein, and/or facilitates purification or detection of said recombinant protein;
- (ii) is truncated from a native N terminal signal sequence found on a corresponding native cutinase; or
- (iii) both (i) and (ii).

7. The isolated recombinant protein of item 6, wherein said carrier protein is YebF as defined by SEQ ID NO:
8.

5 8. A polynucleotide molecule comprising a nucleic acid sequence encoding the recombinant protein as defined in any one of items 1 to 7, operably linked to a heterologous promoter.

9. The polynucleotide of item 8, wherein the recombinant protein encoded by said polynucleotide: (i) lacks an N terminal signal sequence of a native cutinase; (ii) is fused to a heterologous carrier protein; or (iii) both (i) and (ii).

10

10. A vector comprising the polynucleotide molecule as defined in item 8 or 9.

11. A cell comprising the isolated recombinant protein as defined in any one of items 1 to 7, the polynucleotide molecule as defined in item 8 or 9, or the vector as defined in item 9.

15

12. The cell of item 11, which is a bacterial cell, a yeast cell, a non-yeast fungal cell, an insect cell, a plant cell, an animal cell, or a mammalian cell.

13. The cell of item 12, wherein said cell is from *E. coli*, *Bacillus subtilis*, or *Streptomyces* spp.

20

14. A composition comprising: (i) the isolated recombinant protein as defined in any one of items 1 to 7; and (ii) a bleaching agent and/or an organic solvent, wherein said recombinant protein retains said cutinase activity when present in said composition.

25

15. The composition of item 14, wherein:

(i) said bleaching agent is:

(1) an oxidizing agent which is or comprises: hydrogen peroxide, a peroxide other than hydrogen peroxide, a non-peroxide oxidizing agent, ozone, sodium percarbonate, sodium perborate, or any combination thereof; and/or

30

(2) a reducing agent which is or comprises: sodium dithionite, sodium dithionite, sulfur dioxide, a sulfite, a bisulfite, a sodium borohydride, or any combination thereof;

(ii) said organic solvent which is or comprises: methanol, 2-propanol, or another alcohol, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), or acetone; or

(iii) both (i) and (ii).

16. The composition of item 14 or 15, wherein:

(i) said bleaching agent is present at a concentration of:

(1) at least 100 ppm, 150 ppm, 200 ppm, 250 ppm, 300 ppm, 350 ppm, 400 ppm, 450 ppm, 500 ppm, 550 ppm, 600 ppm, 650 ppm, 700 ppm, 750 ppm, 800 ppm, 850 ppm, 900 ppm, 950 ppm, 1000 ppm, 1500 ppm, 2000 ppm, 2500 ppm, 3000 ppm, 3500 ppm, 4000 ppm, 4500 ppm, 5000 ppm, 5500 ppm, 6000 ppm, 6500 ppm, or 7000 ppm; or

(2) between 100 ppm, 150 ppm, 200 ppm, 250 ppm, 300 ppm, 350 ppm, 400 ppm, 450 ppm, 500 ppm, 550 ppm, 600 ppm, 650 ppm, 700 ppm, 750 ppm, 800 ppm, 850 ppm, 900 ppm, 950 ppm, or 1000 ppm, and 1500 ppm, 2000 ppm, 2500 ppm, 3000 ppm, 3500 ppm, 4000 ppm, 4500 ppm, 5000 ppm, 5500 ppm, 6000 ppm, 6500 ppm, or 7000 ppm;

(ii) said organic solvent is present as a concentration of:

(1) at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% v/v; or

(2) between 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, or 25%, and 30%, 35%, 40%, 45%, or 50% v/v; or

(iii) both (i) and (ii).

17. The composition of any one of items 14 to 16, further comprising: (i) an additional recombinant enzyme which is: a lipase, an esterase, a pectate lyase, a pectinase, a cutinase, a cellulase, a hemicellulose, an amylase, or any combination thereof, or another recombinant enzyme; (ii) a detergent; (iii) a surfactant; (iv) a buffer; (v) a chelator; or (vi) any combination of (i) to (v).

18. The composition of any one of items 14 to 17, which is: (i) a cleaning product, a degreaser, a disinfectant, a bleaching product, or any combination thereof; or (ii) feed or a feed additive.

19. Use of the isolated recombinant protein as defined in any one of items 1 to 7, or the composition as defined in any one of items 14 to 17, for: (i) cleaning, degreasing, disinfecting, bleaching product, biofilm control, or any combination thereof; (ii) for inactivating mycotoxin; (iii) bioscouring or other treatment of fabrics or textiles; (iv) catalyzing trans-esterification reactions; (v) vegetable oil extraction; or (vi) enhanced oil recovery.

20. The use of item 18 for inactivating ochratoxin.

21. A method for producing the recombinant protein as defined in any one of items 1 to 7, said method comprising: (i) culturing a suitable host cell expressing a polynucleotide encoding said recombinant protein, wherein said polynucleotide encodes a truncated recombinant protein lacking an N terminal signal sequence; and (ii) isolating said recombinant protein.

5

22. The method of item 21, wherein said polynucleotide is operably linked to a heterologous promoter.

23. The method of item 21 or 22, wherein said polynucleotide encodes said recombinant protein fused to a heterologous carrier protein.

10

In other implementations, the present description relates to one or more of the following items.

1. A recombinant protein having cutinase activity, said protein comprising a cutinase catalytic domain that inactivates mycotoxin.

2. The protein of item 1, wherein said cutinase catalytic domain inactivates ochratoxin.

15 3. The protein of item 1 or 2, wherein said cutinase catalytic domain inactivates aflatoxin.

4. The protein of any one of items 1 to 3, wherein said cutinase catalytic domain inactivates ochratoxin and aflatoxin.

20 5. The protein of any one of items 1 to 4, wherein said cutinase catalytic domain shares at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to the cutinase catalytic domain of any one of SEQ ID NOs: 2 and 13-20.

6. A recombinant protein having cutinase activity, said protein comprising a cutinase catalytic domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to the cutinase catalytic domain of SEQ ID NO: 2.

25 7. The protein of any one of items 1 to 6, wherein said cutinase catalytic domain shares at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to the cutinase catalytic domain of SEQ ID NO: 2.

8. The protein of any one of items 1 to 6, wherein the cutinase catalytic domain comprises the amino acid sequence of SEQ ID NO: 2 or the cutinase catalytic domain of any one of SEQ ID NOs: 13-20.

30 9. The protein of any one of items 1 to 8, further comprising a polymer binding domain operably linked to said cutinase catalytic domain.

10. The protein of item 9, wherein said polymer binding domain binds to poly(hydroxybutyrate), triacin, tributyrin, trimiristin, tripalmitin, polyvinyl acetate (PVA), polyethylene terephthalate (PET), or polytrimethylene terephthalate (PTT).
11. The protein of item 9 or 10, wherein said polymer binding domain comprises an amino acid sequence
5 sharing at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to the polymer binding domain of any one of SEQ ID NOs: 4 and 13-15.
12. The protein of item 11, wherein said polymer binding domain comprises the amino acid sequence of any one of SEQ ID NOs: 4 and 13-15.
- 10 13. The protein of any one of items 1 to 12, wherein said cutinase domain and said polymer binding domain are operably linked by a linker domain.
14. The protein of item 11, wherein said linker domain is a proline/threonine-rich linker domain.
15. The protein of item 13 or 14, wherein said linker domain comprises an amino acid sequence sharing at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%,
15 or at least 95% sequence identity to: SEQ ID NO: 3; residues 220-247 of SEQ ID NO: 13; residues 226-236 of SEQ ID NO: 14; or residues 228-276 of SEQ ID NO: 15.
16. A polynucleotide encoding the protein of any one of items 1 to 15.
17. A vector comprising the polynucleotide of item 16 operably linked to a heterologous promoter.
18. A cell comprising the vector of item 17.
- 20 19. Use of the protein as defined in any one of items 1 to 15 for inactivating mycotoxin.
20. The use of item 19, wherein said mycotoxin comprises ochratoxin.
21. The use of item 19, wherein said mycotoxin comprises aflatoxin.
22. The use of item 20, wherein said mycotoxin comprises ochratoxin and aflatoxin.
23. Use of the protein of any one of items 1 to 15 for degrading a polymer.
- 25 24. The use of item 23, wherein said polymer is a polyester.
25. The use of item 23, wherein said polymer is: cutin; polycaprolactone (PCL); polylactic acid (PLA); poly(1,3-propylen adipate (PPA); poly(hydroxybutyrate); triacin; tributyrin; trimiristin; tripalmitin; polyvinyl acetate (PVA); polyethylene terephthalate (PET); or polytrimethylene terephthalate (PTT).

26. Use of the protein as defined in any one of items 1 to 15 for the manufacture of: a cleaner and/or degreaser; animal feed; a surface treatment product in the pulp and paper industries; or a product for the degradation and/or control of biofilm.

27. A kit for inactivating mycotoxins, said kit comprising the protein as defined in any one of items 1 to 15; and a
5 suitable container.

28. A method for expressing a transgenic KrCUT protein in a cell, said method comprising expression of a YebF-KrCUT nucleotide in the cell.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of specific embodiments thereof, given by way of example only with reference
10 to the accompanying drawings.

Definitions

In the present description, a number of terms are extensively utilized and they should be given the meaning of the person of skill in the art with a mind willing to understand. Furthermore, in order to provide a clear and
15 consistent understanding of the specification and claims, including the scope to be given to such terms, the following definitions are provided.

The term “**about**” is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value. In general, the terminology “about” is meant to designate a possible variation of up to 10%. Therefore, a variation of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10% of a value is included in the term
20 “about”.

As used herein, “**polynucleotide**” or “**nucleic acid molecule**” refers to a polymer of nucleotides and includes DNA (e.g., genomic DNA, cDNA), RNA molecules (e.g., mRNA), and chimeras thereof. The nucleic acid molecule can be obtained by cloning techniques or synthesized. DNA can be double-stranded or single-stranded (coding strand or non-coding strand [antisense]). Conventional deoxyribonucleic acid (DNA) and ribonucleic acid
25 (RNA) are included in the terms “nucleic acid molecule” and “polynucleotide” as are analogs thereof (e.g., generated using nucleotide analogs, e.g., inosine or phosphorothioate nucleotides). Such nucleotide analogs can be used, for example, to prepare polynucleotides that have altered base-pairing abilities or increased resistance to nucleases. A nucleic acid may comprise only conventional sugars, bases and linkages, as found in RNA and DNA, or may include both conventional components and substitutions.

30 As used herein, the term “**gene**” refers to nucleic acid molecules which may be isolated from chromosomal DNA, and very often include an open reading frame encoding a protein, e.g., polypeptides of the present invention. A gene may include coding sequences, non-coding sequences, introns and/or regulatory sequences, as well known.

As used herein, "**recombinant protein**" refers to a protein produced using cells that do not have, in their native state, an endogenous copy of the DNA able to express the protein. The cells produce the recombinant protein because they have been genetically altered by the introduction of the appropriate isolated nucleic acid sequence. The term "recombinant" also includes reference to a cell, or nucleic acid, or vector, that has been modified by the introduction of a heterologous (exogenous or foreign) nucleic acid or the alteration of a native nucleic acid to a form not native to that cell, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell, express mutants of genes that are found within the native form, or express native genes that are otherwise abnormally expressed, under-expressed or not expressed at all.

As used herein, the terms "**hybridizing**" and "**hybridizes**" are intended to describe conditions for hybridization and washing under which nucleotide sequences at least about 60%, at least about 70%, at least about 80%, more preferably at least about 85%, even more preferably at least about 90%, more preferably at least 95%, more preferably at least 98% or more preferably at least 99% homologous to each other typically remain hybridized to each other. A preferred, non-limiting example of such hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 1X SSC, 0.1% SDS at 50°C, preferably at 55°C, preferably at 60°C and even more preferably at 65°C. Highly stringent conditions include, for example, hybridizing at 68°C in 5x SSC/5x Denhardt's solution / 1.0% SDS and washing in 0.2x SSC/0.1% SDS at room temperature. Alternatively, washing may be performed at 42°C. The skilled artisan will know which conditions to apply for stringent and highly stringent hybridization conditions.

The terms "**identity**" and "**percent identity**" are used interchangeably herein. For the purpose of this invention, it is defined here that in order to determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = number of identical positions/total number of positions (i.e., overlapping positions) x 100). Preferably, the two sequences are the same length. Thus, In accordance with the present invention, the term "identical" or "percent identity" in the context of two or more nucleic acid or amino acid sequences, refers to two or more sequences or subsequences that are the same, or that have a specified percentage of amino acid residues or nucleotides that are the same (e.g., 60% or 65% identity, preferably, 70-95% identity, more preferably at least 95% identity), when compared and aligned for maximum correspondence over a window of comparison, or over a designated region as measured using a sequence comparison algorithm as known in the art, or by manual alignment and visual inspection.

Sequences having, for example, 60% to 95% or greater sequence identity are considered to be substantially identical over their full lengths.

In a related manner, the terms "**homology**" or "**percent homology**", refer to a similarity between two polypeptide sequences, but take into account changes between amino acids (whether conservative or not). As well known in the art, amino acids can be classified by charge, hydrophobicity, size, etc. It is also well known in the art that amino acid changes can be conservative (e.g., they do not significantly affect, or not at all, the function of the protein). A multitude of conservative changes are known in the art, Serine for threonine, isoleucine for leucine, arginine for lysine etc., Thus the term homology introduces evolutionistic notions (e.g., pressure from evolution to a retain function of essential or important regions of a sequence, while enabling a certain drift of less important regions).

An "**isolated polynucleotide**" or "**isolated nucleic acid molecule**" is a nucleic acid molecule (DNA or RNA) that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived.

As used herein, an "**isolated polypeptide**" or "**isolated protein**" is intended to include a polypeptide or protein removed from its native environment.

The term "**variant**" refers herein to a polypeptide, which is substantially similar in structure (e.g., amino acid sequence) to a polypeptide disclosed herein or encoded by a nucleic acid sequence disclosed herein without being identical thereto. Thus, two molecules can be considered as variants even though their primary, secondary, tertiary or quaternary structures are not identical. A variant can comprise an insertion, substitution, or deletion of one or more amino acids as compared to its corresponding native protein. A variant can comprise additional modifications (e.g., post-translational modifications).

As used herein, the term "**mycotoxin**" refers to any toxic product formed in a mold, fungus, and/or yeast that exhibits significant toxicity to a human or animal when ingested or otherwise contacted (e.g., on the skin, in the lungs (inhaled), in the eyes, etc.). Thus, specifically contemplated mycotoxins include aflatoxins (and particularly B1, B2, G1, and G2), fumonisins (and particularly B1, B2, and B3), ochratoxin, deoxynivalenol (DON, vomitoxin), T-2 toxin, and zearalenone.

As used herein the expressions "**inactivates**", "**inactivating**", "**detoxifies**", or "**detoxifying**", in the context of mycotoxins, refers to the processing of a mycotoxin in accordance with the present invention such that an undesirable effect of the mycotoxin is reduced or eliminated. In some embodiments, such undesirable effects may include reduction or elimination of toxicity to a human or animal when the processed mycotoxin is ingested or otherwise contacted (e.g., on the skin, in the lungs (inhaled), in the eyes, etc.).

Other definitions may be provided throughout the description.

BRIEF DESCRIPTION OF THE DRAWINGS

In the appended drawings:

Figure 1 is a schematic representation of the domain organization of KrCUT and sequence characteristics of some conserved regions. SP, signal peptide; T/P-rich, threonine-proline rich linker; "KrCUT206" (containing 206 amino acids) is a truncated version of KrCUT lacking the polymer-binding domain. "KrCUT189" (containing 189 amino acids) is a truncated version of KrCUT206 further lacking the T/P-rich linker. Inset **(a)**: "cutinase motifs" containing the predicted catalytic triads (S114-D184-H198) and conserved cysteines (C103, C180, C187) according to Wang et al., 2010. Inset **(b)**: Homology to substrate-binding domains of PHB depolymerases. Abbreviations of bacterial source: Cf, *Cellulomonas flavigena* DSM 20109 (ZP_04367703); Ct, *Comamonas testosteroni* (BAA22882); Fs, *Fusarium solani* (P00590); Kr, *Kineococcus radiotolerans* cutinase (YP_001363838); Mf, *Monilinia fructicola* (AAZ95012); Mg, *Magnaporthe grisea* (CAA43717); Nd, *Nocardiopsis dassonvillei* subsp PHB depolymerase (ZP_04334644); Pi, *Phytophthora infestans* (AAY43367); Pl, *Paucimonas lemoignei* (AAB48166); Rh, *Rhodococcus (jostii)* RHA1 (YP_700622). Tf, *Thermobifida fusca* YX PHB depolymerase (YP_288143, re-annotated as WP_011290529.1); and Ts, *Thermobifida* sp. BCC23166 PHB depolymerase (ACF17837).

Figure 2 shows purification of recombinant *K. radiotolerans* cutinase and truncated derivatives. Proteins were analyzed by 10% SDS PAGE, and silver stained. The molecular weight markers are indicated alongside; **(a)** lane 1, fraction from SP-sepharose™ showing YebF-KrCUT fusion protein (39 kDa) and mature KrCUT (31 kDa); lanes 2 and 3 from HiLoad™ Superdex™ 75 showing the separated protein bands; **(b)** lane 4, YebF-KrCUT189 (33 kDa) from HiLoad™ Superdex™ 75.

Figure 3 shows substrate specificity of full-length KrCUT and KrCUT189. Activity (U/mg) towards selected *p*-nitrophenol esters are compared.

Figure 4 shows pH optima curves for activity of KrCUT and KrCUT189.

Figure 5 shows thermostability curves for KrCUT. Activity of the enzyme after incubation at the defined temperatures and for the selected time periods was measured.

Figure 6 shows the enzymatic activity of protein purification elution fractions corresponding to KrCUT (fractions C1-C10) and YebF-KrCUT (fractions D9-D11).

Figure 7 shows GC analysis of cutin monomers released by KrCUT. The numbers on the peaks correspond to the retention times of the released products as described in the text.

Figure 8 shows degradation of synthetic polyesters by YebF-KrCUT and KrCUT189. Enzyme concentration is 2.5 μM, unless otherwise stated. **(A)** Degradation of PCL films (■, YebF-KrCUT; ▲, YebF-KrCUT, 1.25 μM; and □, KrCUT189), and PCL pellets (●, YebF-KrCUT; and ○, KrCUT189). The curve of PCL film degradation by 5 μM YebF-KrCUT approximated that of 2.5 μM enzyme concentration (not shown). **(B)** Degradation of PLA (●, YebF-KrCUT; and ○, KrCUT189) and polypropylene adipate (■, YebF-KrCUT; and □, KrCUT189).

Figure 9 shows adsorption isotherm of cutinase and PHB granules at 25 °C. Purified KrCUT of concentrations ranging from 0.16 to 3.2 mg/mL were mixed with 25 mg PHB in 50 mM sodium phosphate (pH 8.0) in a total volume of 1 mL and mixed by gently shaking for 3 h. The PHB polymer was removed by centrifugation and the concentration of protein in the supernatant was determined by the BCA method. The concentration of bound protein at a particular concentration of cutinase was calculated as the difference between a control without added PHB and free cutinase after incubation with PHB.

Figure 10 shows amounts of pectin released from natural hemp fibres by pectate lyase (PL) or in combination with cutinase (Cut) at different concentrations. The shaded bars indicate the two different incubation periods (1.5 h and 3 h).

Figure 11 (A) and (B) show light microscopic analysis of hemp fibre obtained by enzymatic retting. **(A)** pectate lyase treatment, fibers are embedded into pectin and wax material; **(B)** pectate lyase and cutinase treatment, fiber, F cell in bundles. **(C)** and **(D)** represents images from scanning electron microscopy (SEM): **(C)** pectate lyase; **(D)** pectate lyase and cutinase.

Figure 12 shows the long term stability of recombinant KrCUT in stabilisation buffer.

Figure 13 shows examples of purified recombinant proteins separated by SDS-PAGE. Panel (A) shows the enzyme KrCUT, while panel (B) shows the variant "Am-Kr" alone or fused to the heterologous carrier protein, YebF ("YebF-Am-Kr").

Figure 14 shows CD spectroscopy measurements in order to monitor the unfolding/refolding of various recombinant proteins: (A) KrCUT; (B) Am-Kr; (C); CfCUT; and (D) Cf-TP.

Figure 15 shows a multiple sequence alignment (A) and a phylogenetic tree (B) of native multi-domain cutinases from *Kineococcus radiotolerans*, *Cellulomonas bogoriensis*, *Cellulomonas cellasea*, and *Cellulomonas flavigena*.

SEQUENCE LISTING

This application contains a Sequence Listing in computer readable form entitled Sequence_Listing.txt, created January 28, 2016 having a size of 84 kb. The computer readable form is incorporated herein by reference.

SEQ ID NO: 1	ssKrCUT amino acid sequence	SEQ ID NO: 25	ssCbCUT2: cutinase from Cb 69B4 (KGM14008.1) amino acid sequence
SEQ ID NO: 2	KrCUT189 amino acid sequence (cutinase catalytic domain of KrCUT)	SEQ ID NO: 26	AmCUT: cutinase from Am ATCC 39116 amino acid sequence
SEQ ID NO: 3	T/P-rich linker of KrCUT amino acid sequence	SEQ ID NO: 27	Am-Kr: variant amino acid sequence
SEQ ID NO: 4	Polymer binding domain of KrCUT amino acid sequence	SEQ ID NO: 28	CfCUT: cutinase from Cf amino acid sequence
SEQ ID NO: 5	DNA encoding KrCUT optimized for expression in <i>E. coli</i>	SEQ ID NO: 29	Cf-TP: variant amino acid sequence
SEQ ID NO: 6	KrCUT amino acid sequence, lacking residues 1-33 of ssKrCUT	SEQ ID NO: 30	cutinase catalytic domain of CfCUT amino acid sequence

SEQ ID NO: 7	DNA encoding YebF	SEQ ID NO: 31	T/P-rich linker domain of CfCUT amino acid sequence
SEQ ID NO: 8	YebF amino acid sequence	SEQ ID NO: 32	polymer binding domain of CfCUT amino acid sequence
SEQ ID NO: 9	DNA encoding YebF-KrCUT	SEQ ID NO: 33	cutinase catalytic domain of CbCUT1 (KGM14009.1) amino acid sequence
SEQ ID NO: 10	YebF-KrCUT amino acid sequence	SEQ ID NO: 34	linker of CbCUT1 (KGM14009.1) amino acid sequence
SEQ ID NO: 11	DNA encoding YebF-KrCUT189	SEQ ID NO: 35	polymer binding domain of CbCUT1 (KGM14009.1) amino acid sequence
SEQ ID NO: 12	YebF-KrCUT189 amino acid sequence	SEQ ID NO: 36	cutinase catalytic domain of CbCUT2 (KGM14008.1) amino acid sequence
SEQ ID NO: 13	ssCfCUT: cutinase from <i>Cf</i> amino acid sequence	SEQ ID NO: 37	T/P-rich linker of CbCUT2 (KGM14008.1) amino acid sequence
SEQ ID NO: 14	ssCbCUT1: cutinase from <i>Cb</i> amino acid sequence	SEQ ID NO: 38	polymer binding domain of CbCUT2 (KGM14008.1) amino acid sequence
SEQ ID NO: 15	ssCcCUT: cutinase from <i>Cc</i> amino acid sequence	SEQ ID NO: 39	cutinase catalytic domain of CcCUT (DSM 20118) amino acid sequence
SEQ ID NO: 16	ssAmCUT: cutinase from <i>Am</i> ATCC 39116 amino acid sequence	SEQ ID NO: 40	T/P-rich linker of CcCUT (DSM 20118) amino acid sequence
SEQ ID NO: 17	ssFrCUT: cutinase from <i>Fr</i> amino acid sequence	SEQ ID NO: 41	polymer binding domain of CcCUT (DSM 20118) amino acid sequence
SEQ ID NO: 18	ssAcCUT: cutinase from <i>Ac</i> 431 amino acid sequence	SEQ ID NO: 42	cutinase catalytic domain of FrCUT (BMG5.12) amino acid sequence
SEQ ID NO: 19	ssCaCUT: cutinase from <i>Ca</i> amino acid sequence	SEQ ID NO: 43	cutinase catalytic domain of AcCUT (431) amino acid sequence
SEQ ID NO: 20	ssRhCUT: cutinase from <i>Rh</i> amino acid sequence	SEQ ID NO: 44	cutinase catalytic domain of CaCUT (DSM 44712) amino acid sequence
SEQ ID NO: 21	CfKr fusion protein amino acid sequence	SEQ ID NO: 45	cutinase catalytic domain of RhCUT amino acid sequence
SEQ ID NO: 22	DNA encoding CfKr fusion protein	SEQ ID NO: 46	CbCUT2: cutinase from <i>Cb</i> (KGM14008.1) amino acid sequence
SEQ ID NO: 23	KrCf fusion protein amino acid sequence	SEQ ID NO: 47	CcCUT: cutinase from CcCUT (DSM 20118) amino acid sequence
SEQ ID NO: 24	DNA encoding KrCf fusion protein	SEQ ID NO: 48	CbCUT1: cutinase from CbCUT1 (KGM14009.1) amino acid sequence

ss = native N-terminal signal sequence

Kr = *Kineococcus radiotolerans*Cf = *Cellulomonas flavigena*Cb = *Cellulomonas bogoriensis*Cc = *Cellulomonas cellasea*Am = *Amycolatopsis* sp.Fr = *Frankia* sp. BMG5.12Ac = *Actinoplanes missouriensis*Ca = *Cryptosporangium arvum*Rh = *Rhodococcus*TP = threonine/proline-rich linker domain from *Kr*

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Recombinant proteins

In one aspect, the present description relates to an isolated multi-domain recombinant protein having cutinase activity. The multi-domain recombinant protein may comprise: (a) a cutinase catalytic domain; (b) a proline/threonine-rich linker domain positioned C-terminal of domain (a); and (c) a polymer binding domain positioned C-terminal of domain (b).

As used herein, the expression "**cutinase catalytic domain**" refers to a polypeptide domain that has the ability to hydrolyze cutin (and/or another polymer substrate of interest hydrolyzable by the cutinase catalytic domain) in a modular fashion (i.e., when present within a larger recombinant polypeptide of the present invention).

In some embodiments, the cutinase catalytic domain may be: (1) a cutinase catalytic domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity of SEQ ID NOs: 2, 30, 33, 36, 39, or 42-45; (2) a cutinase catalytic domain comprising the amino acid sequence of SEQ ID NOs: 2, 30, 33, 36, 39, or 42-45; or (3) both (1) and (2).

In some embodiments, the cutinase catalytic domain may share at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to the cutinase catalytic domain of any one of SEQ ID NOs: 2 and 13-21.

As used herein, the expression "**threonine/proline-rich linker domain**" or "**proline/threonine-rich linker domain**" (T/P-rich linker domain) refers to a polypeptide domain that shares functional and/or structural similarity to T/P-rich linker domains found in native multi-domain cutinases, such as those described herein from *Kineococcus radiotolerans* (e.g., accession ABS05574.1), *Cellulomonas flavigena* (e.g., accession ADG75999.1), *Cellulomonas bogoriensis* (e.g., accessions KGM14008.1), and *Cellulomonas cellasea* (e.g., accession KGM03336.1).

In some embodiments, the T/P-rich linker domain may be a threonine/proline-rich linker domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to SEQ ID NO: 3, 31, 34, 37, or 40. In some embodiments, the T/P-rich linker domain may be a T/P-rich linker domain comprising the amino acid sequence of SEQ ID NO: 3, 31, 34, 37, or 40.

In some embodiments, the T/P-rich linker domain may be a polypeptide domain which has at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 50%, at least 55%, at least 60%, or at least 65% of its residues being a proline or a threonine, over a stretch of 15 to 55 consecutive amino acids.

In some embodiments, the linker domain may comprise an amino acid sequence sharing at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to: SEQ ID NO: 3; residues 220-247 of SEQ ID NO: 13; residues 226-236 of SEQ ID NO: 14; or residues 228-276 of SEQ ID NO: 15.

As used herein, the expression "**polymer binding domain**" refers to a protein domain that may have the ability to bind in a modular fashion (i.e., when present within a larger recombinant polypeptide of the present

description) to a given polymer of interest (e.g., a polyester hydrolyzable by a cutinase catalytic domain of the present description).

In some embodiments, the polymer binding domain may have at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to SEQ ID NO: 4, 32, 35, 38, or 41. In some embodiments, the polymer binding domain may comprise the amino acid sequence of SEQ ID NO: 4, 32, 35, 38, or 41.

In some embodiments, the polymer binding domain of a polypeptide of the present description may bind to poly(hydroxybutyrate), triacin, tributyrin, trimiristin, tripalmitin, polyvinyl acetate (PVA), polyethylene terephthalate (PET), or polytrimethylene terephthalate (PTT). In some embodiments, the polymer binding domain may comprise an amino acid sequence sharing at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to the polymer binding domain of any one of SEQ ID NOs: 4 and 13-15. In some embodiments, the polymer binding domain comprises the amino acid sequence of any one of SEQ ID NOs: 4 and 13-15.

In some embodiments, an isolated recombinant protein of the present description may be a chimeric recombinant protein, wherein at least one of domains (a), (b) and (c) is from a cutinase belonging to a different species. Native multi-domain cutinases can be found in species such as *Kineococcus radiotolerans* (e.g., accession ABS05574.1), *Cellulomonas flavigena* (e.g., accession ADG75999.1), *Cellulomonas bogoriensis* (e.g., accessions KGM14008.1 and KGM14009.1), or *Cellulomonas cellasea* (e.g., accession KGM03336.1).

In some embodiments, the above mentioned protein further comprises a polymer binding domain operably linked to the cutinase catalytic domain. As used herein, the expression "**operably linked**" refers to the covalent linkage of two or more modular protein domains (e.g., a cutinase catalytic domain and a polymer binding domain) such that each of the modular protein domains at least substantially retains its enzymatic activity. Two or more protein domains that cooperate synergistically when covalently linked is also within the meaning of "operably linked" as used herein.

In some embodiments, the above mentioned cutinase domain and the above mentioned polymer binding domain are operably linked by a linker domain. In some embodiments, the linker domain is 5 to 60, 5 to 50, 5 to 40, 5 to 30, 5 to 20, 10 to 60, 10 to 50, or 10 to 40 amino acids in length. In some embodiments, the linker domain is a proline/threonine-rich linker domain. As used herein, the expression "**proline/threonine-rich**" refers to a polypeptide domain which has at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 50%, at least 55%, at least 60%, or at least 65% of its residues being a proline or a threonine. In some embodiments, the linker domain comprises an amino acid sequence sharing at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to: SEQ ID NO: 3; residues 220-247 of SEQ ID NO: 13; residues 226-236 of SEQ ID NO: 14; or residues 228-276 of SEQ ID NO: 15.

In another aspect, the present invention relates to isolated recombinant proteins sharing a minimum threshold of amino acid sequence identity with any one of the polypeptides as described herein. In specific embodiments, the present invention relates to isolated polypeptides having at least 60%, 65%, 70%, 71%, 72, 73%, 74%, 75%, 76%, 77%, 78%, 79, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% amino acid sequence identity to any one of the above-mentioned polypeptides. Other specific percentage units that have not been specifically recited here for brevity are nevertheless considered within the scope of the present invention.

In some embodiments, the isolated recombinant protein of the present description may have **improved stability to a bleaching agent**, as compared to a native cutinase enzyme that comprises the corresponding cutinase catalytic domain. As used herein, a **"bleaching agent"** refers to an agent that lightens or whitens a substrate through chemical reaction. Bleaching reactions usually involve oxidative or reductive processes that degrade color. As used in the present context, **"increased stability"** refers to an enzyme that generally retains a higher relative level of enzymatic activity, or exhibits a lower relative loss of enzymatic activity, after being exposed to increasing concentrations of a given bleaching agent, as compared to a suitable control enzyme (e.g., native enzyme).

In some embodiments, the bleaching agent may be an oxidizing agent or a reducing agent. The oxidizing agent may include hydrogen peroxide, a peroxide other than hydrogen peroxide, a non-peroxide oxidizing agent, ozone, sodium percarbonate, sodium perborate, or any combination thereof. The reducing agent may include sodium dithionite, sodium dithionite, sulfur dioxide, a sulfite, a bisulfite, a sodium borohydride, or any combination thereof.

In some embodiments, the isolated recombinant protein of the present description retains activity in the presence of a chelator (e.g., EDTA or GLDA). In some embodiments, the isolated recombinant protein of the present description retains activity in the presence of EDTA at a concentration of between 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, or 9%, and 10% w/v). In some embodiments, the isolated recombinant protein of the present description retains activity in the presence of GLDA at a concentration between 0.5%, 1%, 2%, 3%, or 4%, and 5% w/v).

In some embodiments, the isolated recombinant protein of the present description may have **improved stability to an organic solvent** (e.g., methanol, 2-propanol, or another alcohol, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), acetone, or any combination thereof), as compared to a native cutinase enzyme that comprises the corresponding cutinase catalytic domain. As used in the present context, **"increased stability"** refers to an enzyme that generally retains a higher relative level of enzymatic activity, or exhibits a lower relative loss of enzymatic activity, after being exposed to increasing concentrations of a given organic solvent, as compared to a suitable control enzyme (e.g., native enzyme).

In some embodiments, the isolated recombinant protein of the present description may exhibit a broader temperature activity range, as compared to a native cutinase enzyme that comprises the corresponding cutinase catalytic domain. Such properties may increase the versatility of an enzyme for industrial applications. As used in the

present context, “**broader temperature activity range**” refers to an enzyme that generally retains a higher relative level of enzymatic activity, or exhibits a lower relative loss of enzymatic activity, when incubated across a broader range of reaction temperatures, as compared to a suitable control enzyme (e.g., native enzyme). This is in contrast to an enzyme that, for example, shows a maximal level of activity at a given optimal temperature, but exhibits a relatively sharp decrease in activity when the reaction temperature is progressively shifted away from that optimal temperature.

It will be appreciated that this disclosure embraces the degeneracy of codon usage as would be understood by one of ordinary skill in the art and as illustrated in the table below.

10

Codon Degeneracies	
Amino Acid	Codons
Ala/A	GCT, GCC, GCA, GCG
Arg/R	CGT, CGC, CGA, CGG, AGA, AGG
Asn/N	AAT, AAC
Asp/D	GAT, GAC
Cys/C	TGT, TGC
Gln/Q	CAA, CAG
Glu/E	GAA, GAG
Gly/G	GGT, GGC, GGA, GGG
His/H	CAT, CAC
Ile/I	ATT, ATC, ATA
Leu/L	TTA, TTG, CTT, CTC, CTA, CTG
Lys/K	AAA, AAG
Met/M	ATG
Phe/F	TTT, TTC
Pro/P	CCT, CCC, CCA, CCG
Ser/S	TCT, TCC, TCA, TCG, AGT, AGC
Thr/T	ACT, ACC, ACA, ACG
Trp/W	TGG
Tyr/Y	TAT, TAC
Val/V	GTT, GTC, GTA, GTG
START	ATG
STOP	TAG, TGA, TAA

Conservative substitutions: Furthermore, it will be understood by one skilled in the art that conservative substitutions may be made in the amino acid sequence of a polypeptide without disrupting the structure or function of the polypeptide. Conservative substitutions are accomplished by the skilled artisan by substituting amino acids with similar hydrophobicity, polarity, and R-chain length for one another. Additionally, by comparing aligned

sequences of homologous proteins from different species, conservative substitutions may be identified by locating amino acid residues that have been mutated between species without altering the basic functions of the encoded proteins. The table below provides an exemplary list of conservative substitutions.

Conservative Substitutions

Type of Amino Acid	Substitutable Amino Acids
Hydrophilic	Ala, Pro, Gly, Glu, Asp, Gln, Asn, Ser, Thr
Sulphydryl	Cys
Aliphatic	Val, Ile, Leu, Met
Basic	Lys, Arg, His
Aromatic	Phe, Tyr, Trp

In another embodiment, the present invention relates to functional variants of the recombinant proteins as described herein. **“Functional variants”** refers to recombinant proteins differing from their parent recombinant protein by one or more amino acid substitutions, deletions, and/or insertions, yet which at least retain or provide higher biological activity as compared to their parent recombinant protein. Functional variants include **functional fragments** (i.e., biologically active fragments) of any one of the recombinant polypeptides of the present invention. Such fragments include fewer amino acids than the full length protein from which they are derived, but exhibit at least one biological activity of the corresponding full-length protein.

In another aspect, the present invention includes **fusion proteins** comprising a polypeptide of the present invention or a functional variant thereof, which is operatively linked to one or more unrelated polypeptide (e.g., heterologous amino acid sequences). **“Unrelated polypeptides”** or **“heterologous polypeptides”** or **“heterologous sequences”** refer to polypeptides or sequences which are usually not present close to or fused to one of the polypeptides of the present invention. Such “unrelated polypeptides” or “heterologous polypeptides” having amino acid sequences corresponding to proteins which are not substantially homologous to the polypeptide sequences disclosed herein. Such “unrelated polypeptides” can be derived from the same or a different organism. In one embodiment, a fusion protein of the present invention comprises at least two biologically active portions or domains of polypeptide sequences disclosed herein.

In another embodiment, a recombinant protein of the present invention can be fused to a protein which enables or facilitates recombinant protein purification and/or detection. For example, a polypeptide of the present invention can be fused to a protein such as glutathione S-transferase (GST), and the resulting fusion protein can then be purified/detected through the high affinity of GST for glutathione. In another embodiment, a recombinant protein of the present invention can be fused or otherwise operably linked to a carrier protein (e.g., YebF) which enables its extracellular secretion. Such a linkage can be made such the carrier protein is cleavable upon secretion of the recombinant protein of the present invention.

Recovery and purification

In another aspect, polypeptides of the present invention may be present alone (e.g., in an isolated or purified form), within a composition including a carrier (e.g., an enzymatic composition for carrying out an industrial process), or in an appropriate host. In one embodiment, polypeptides of the present invention can be recovered and purified from cell cultures (e.g., recombinant cell cultures) by methods known in the art. In another embodiment, high performance liquid chromatography ("HPLC") can be employed for the purification.

In another aspect, polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, fungal, plant, insect, animal and mammalian cells. Depending on the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

Polynucleotides

In some aspects, the present description relates to polynucleotides encoding the recombinant proteins described herein. In some embodiments, the recombinant protein encoded by said polynucleotide may lack an N terminal signal sequence often found on native cutinases. The recombinant truncation of such native N terminal signal sequences may increase the enzymatic activity, and thus the utility of the recombinant enzyme.

In some embodiments, the polynucleotides of the present description may encode a recombinant protein described herein which is fused to a heterologous carrier protein (e.g., YebF) and/or a heterologous tag (e.g., a purification or detection tag). Such fusions may not only enable the proper expression and/or secretion of the recombination protein from a host production cell, but also may facilitate the autolysis of recombination proteins over time, which may result in truncated enzyme having higher activity and/or increased structural stability.

In some embodiments, the polynucleotides described here may be fused to a heterologous promoter to improve their expression in a host production cell.

In some aspects, the present invention relates to a polynucleotide encoding a recombinant protein as described herein. In some embodiments, the polynucleotide may be RNA or DNA (e.g., cDNA).

In another aspect, the present invention relates to isolated polynucleotides sharing a minimum threshold of nucleic acid sequence identity with any one of the polynucleotides as described herein. In specific embodiments, the present invention relates to isolated polynucleotides having at least 60%, 65%, 70%, 71%, 72, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% nucleic acid sequence identity to any one of the polynucleotides as described herein. Other specific percentage units that have not been specifically recited here for brevity are nevertheless considered within

the scope of the present invention. Polynucleotides having the aforementioned thresholds of nucleic acid sequence identity can be created by introducing one or more nucleotide substitutions, additions or deletions into the coding nucleotide sequences of the present invention such that one or more amino acid substitutions, deletions or insertions are introduced into the encoded polypeptide. Such mutations may be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis.

In some aspects, the present invention relates to a vector comprising a polynucleotide as described herein operably linked to a heterologous promoter. As used herein in the context of polynucleotides, the term "**heterologous**" refers to the combination of two or more polynucleotide molecules when such a combination is not normally found in nature. For example, the two molecules may be derived from different species and/or the two molecules may be derived from different genes, e.g. different genes from the same species or the same genes from different species. A promoter is thus heterologous with respect to an operably linked transcribable polynucleotide molecule if such a combination is not normally found in nature, i.e., that transcribable polynucleotide molecule is not naturally occurring operably linked in combination with that promoter molecule. In some embodiments, the promoter may be a constitutively active promoter, or may be an inducible promoter. In some embodiments, the promoter may be a viral promoter, bacterial cell promoter, a yeast cell promoter, a fungal cell promoter, an insect cell promoter, an animal cell promoter, or a mammalian cell promoter. A heterologous promoter may be a synthetic promoter that differs from an endogenous promoter by the addition, deletion or substitution of one or more nucleotides.

Vectors

Another aspect of the invention pertains to vectors (e.g., expression vectors), containing a polynucleotide encoding a polypeptide of the present invention. As used herein, the term "**vector**" includes a nucleic acid molecule capable of transporting another nucleic acid molecule to which it has been linked.

Cells

In another aspect, the present description features cells, e.g., transformed host cells or recombinant host cells that contain a polynucleotide or vector described herein. A "**transformed cell**" or "**recombinant cell**" is a cell into which (or into an ancestor of which) has been introduced a polynucleotide or vector of the present description by means of recombinant DNA techniques. Both prokaryotic and eukaryotic cells may be included, e.g., a bacterial cell, a yeast cell, a non-yeast fungal cell (e.g., a filamentous fungus), an insect cell, a plant cell, an animal cell, or a mammalian cell. In some embodiments, the cells may be from *E. coli*, *Bacillus subtilis*, or *Streptomyces* spp.

Expression of Transgenic Proteins

Several experiments were conducted trying to express KrCUT as well as other recombinant proteins in *E. coli*. Various vectors were tested with the codon-optimized gene of krCUT including and excluding the enzyme's

natural signal sequence to express the protein intracellular. Extensive growth and induction conditions were tested but all experiments yielded very low or no expression of the protein.

However, it has presently been found that expression of the KrCUT protein could be achieved by the fusion of KrCUT to the YebF protein. This strategy yielded reproducible protein expression and secretion.

5 The active proteins (confirmed by N-terminal sequencing) have an insertion of two amino acids (leucine and glutamine) that have been introduced as linkers between YebF and the natural occurring KrCUT and KrCUT206 sequence, respectively. In the mature expressed protein, those two amino acids are not cleaved off through the autocatalytic action of the enzyme but remain on the protein's N-terminus. Monitoring the protein after production reveals a cleavage of the N-terminal fused YebF protein which also results in an increase in enzyme activity (about 5 times). At the same time this N-terminal protein cleavage appears to be a slow process which is not related to protease activity but rather to autocatalytic protein hydrolysis as it occurs also in a purified protein solution.

10 Protein fractions after purification on SP-Sepharose™ contain KrCUT or other recombinant enzymes described herein with and without intact YebF-N-terminal fusion, which can be separated using size exclusion chromatography. Over time, the proteins undergo a change in the protein patterns by completely losing the fusion protein YebF. After storage for several weeks at 4°C. The only protein band left is KrCUT (or other recombinant proteins lacking the YebF carrier protein) showing a single protein band which consists now exclusively of the truncated recombinant protein.

Uses & Compositions

20 In some aspects, the present description relates to a composition comprising (i) the isolated recombinant protein as defined herein; and (ii) an additive. As used herein, an "**additive**" refers to an agent that, when added to a composition of the present description, increases performance, stability, and/or shelf-life, as compared to a composition comprising the recombinant protein alone.

25 In some aspects, the present description relates to a composition comprising (i) the isolated recombinant protein as defined herein; and (ii) a bleaching agent and/or an organic solvent, wherein said recombinant protein retains said cutinase activity when present in said composition.

30 In some embodiments, the composition may be a "ready to use" formulation wherein the bleaching agent may be present at a concentration of at least 100 ppm, 150 ppm, 200 ppm, 250 ppm, 300 ppm, 350 ppm, 400 ppm, 450 ppm, 500 ppm, 550 ppm, 600 ppm, 650 ppm, 700 ppm, 750 ppm, 800 ppm, 850 ppm, 900 ppm, 950 ppm, 1000 ppm, 1500 ppm, 2000 ppm, 2500 ppm, 3000 ppm, 3500 ppm, 4000 ppm, 4500 ppm, 5000 ppm, 5500 ppm, 6000 ppm, 6500 ppm, or 7000 ppm. In some embodiments, the bleaching agent may be present at a concentration of between 100 ppm, 150 ppm, 200 ppm, 250 ppm, 300 ppm, 350 ppm, 400 ppm, 450 ppm, 500 ppm, 550 ppm, 600 ppm, 650 ppm, 700 ppm, 750 ppm, 800 ppm, 850 ppm, 900 ppm, 950 ppm, or 1000 ppm, and 1500 ppm, 2000 ppm, 2500 ppm, 3000 ppm, 3500 ppm, 4000 ppm, 4500 ppm, 5000 ppm, 5500 ppm, 6000 ppm, 6500 ppm, or 7000 ppm.

In some embodiments, the composition may be a dilutable "concentrate" wherein the bleaching agent may be present at a concentration of at least 3%, 4%, 5%, or 6% v/v.

5 In some embodiments, the bleaching agent may be an oxidizing agent or a reducing agent. The oxidizing agent may include hydrogen peroxide, a peroxide other than hydrogen peroxide, a non-peroxide oxidizing agent, ozone, sodium percarbonate, sodium perborate, or any combination thereof. The reducing agent may include sodium dithionite, sodium dithionite, sulfur dioxide, a sulfite, a bisulfite, a sodium borohydride, or any combination thereof.

10 In some embodiments, the organic solvent may be: methanol, 2-propanol, or another alcohol, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), or acetone. In some embodiments, the organic solvent may be present at a concentration of at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% v/v. In some embodiments, the organic solvent may be present at a concentration of between 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, or 25%, and 30%, 35%, 40%, 45%, or 50% v/v. In some embodiments, the organic solvent is DMSO and is present at a maximum concentration of 50% v/v. In some embodiments, the organic solvent is acetone and is present at a maximum concentration of 30% v/v.

15 In some embodiments, the compositions of the present description may comprise one or more an additional recombinant enzyme(s). Examples of such additional recombinant enzymes may include: a lipase, an esterase, a pectate lyase, a pectinase, a cutinase, a cellulase, a hemicellulose, an amylase, or any combination thereof. In some embodiments, the composition does not comprise a protease that degrades or inactivates a recombinant protein as defined herein. In some embodiments, the composition comprises a protease and a stabilizer (e.g., a polyol and/or boric acid).

20 In some embodiments, the compositions of the present description may comprise one or more additives such as a detergent; a surfactant; a buffer; a chelator (e.g., EDTA or GLDA); a stabilizer; or any combination thereof. In some embodiments, the composition comprises EDTA (ethylenediaminetetraacetic acid) at a concentration of between 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, or 9%, and 10% w/v. In some embodiments, the composition comprises GLDA (L-Glutamic acid N,N-Diacetic acid Tetra sodium) at a concentration between 0.5%, 1%, 2%, 3%, or 4%, and 5% w/v). In some embodiments, the composition comprises GLDA at a concentration of at least 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% w/v). In some embodiments, the composition comprises GLDA at a concentration of at least 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% w/v).

30 In some embodiments, the compositions may comprise an additive which is a stabilization buffer comprising one or more of sodium gluconate (e.g., 1-5% v/v), sodium carbonate (e.g., 1-5% v/v), sodium citrate (e.g., 1-5% v/v), and propylene glycol (e.g., 1-5% v/v). In some embodiments, the compositions may comprise a stabilization buffer comprising one or more of a polyol such as propylene glycol and glycerol, boric acid and boron derivatives, and sodium formate. In some embodiments, the composition comprises a polyol and/or boric acid. In some

embodiments, the compositions of the present description may be: a cleaning product, a degreaser, a disinfectant, a bleaching product, or any combination thereof; or feed or a feed additive.

In some embodiments, the present description relates to the use of the isolated recombinant protein as defined herein, or the composition as defined herein, for: (i) cleaning, degreasing, disinfecting, bleaching, biofilm control, or any combination thereof; (ii) for inactivating mycotoxin (e.g., ochratoxin and/or aflatoxin); (iii) bioscouring or other treatment of fabrics or textiles; (iv) catalyzing trans-esterification reactions; (v) vegetable oil extraction; or (vi) enhanced oil recovery.

In some embodiments, the present description relates to the isolated recombinant protein as defined herein, or the composition as defined herein, for use in: (i) cleaning, degreasing, disinfecting, bleaching, biofilm control, or any combination thereof; (ii) for inactivating mycotoxin; (iii) bioscouring or other treatment of fabrics or textiles; (iv) catalyzing trans-esterification reactions; (v) vegetable oil extraction; or (vi) enhanced oil recovery.

In some embodiments, the present description relates to the use of the isolated recombinant protein as defined herein, or the composition as defined herein, for the manufacture of an industrial product for: (i) cleaning, degreasing, disinfecting, bleaching, biofilm control, or any combination thereof; (ii) for inactivating mycotoxin; (iii) bioscouring or other treatment of fabrics or textiles; (iv) catalyzing trans-esterification reactions; (v) vegetable oil extraction; or (vi) enhanced oil recovery.

In some embodiments, the present description relates to a kit comprising a recombinant protein as defined herein and a suitable container. In some embodiments, the kit may be for one or more of the above mentioned uses.

In some aspects, the present invention relates to the use of a recombinant protein as described herein for inactivating mycotoxin.

In some aspects, polypeptides of the present invention may be useful for mycotoxin detoxification (mycotoxin inactivation). Advantageously, and unlike some other fungal cutinases that may have been used for mycotoxin detoxification, polypeptides of the present invention may be useful for inactivating more than one mycotoxin. In some embodiments, polypeptides of the present invention may be useful for inactivating Ochratoxin. In some embodiments, polypeptides of the present invention may be useful for inactivating Aflatoxin. In some embodiments, polypeptides of the present invention may be useful for inactivating both Ochratoxin and Aflatoxin (e.g., from the fungi strains *Aspergillus flavus* and/or *Aspergillus niger*).

In some aspects, the present invention relates to a kit for inactivating mycotoxins, said kit comprising a recombinant protein of the present invention as defined herein; and a suitable container.

In some aspects, the present invention relates to the use of a recombinant protein as described herein for degrading a polymer. In some embodiments the polymer is a homopolymer or a heteropolymer. In some embodiments, the polymer is a polyester. In some embodiments, the polymer is: cutin; polycaprolactone (PCL); polylactic acid (PLA); poly(1,3-propylene adipate (PPA); poly(hydroxybutyrate); triacin; tributyrin; trimiristin; tripalmitin; polyvinyl acetate (PVA); polyethylene terephthalate (PET); or polytrimethylene terephthalate (PTT).

In other embodiments, polypeptides of the present invention may be employed with one or more other enzymes (e.g., pectate lyase) for the degradation/hydrolysis of a polymer of interest.

Applications of the recombinant proteins of the present invention in industries such as food, laundry/detergent, textiles, recycling, and/or polymer manufacturing are also envisioned. In some aspects, the present invention may relate to the use of a recombinant protein as described herein in cleaners/degreasers (industrial and/or domestic); animal feed; the treatment of surface in the pulp and paper industries; and/or in the degradation/control of biofilm.

The present invention is illustrated in further details by the following non-limiting examples.

Example 1:

Material and Methods

1.1 Reagents and chemicals. *p*-Nitrophenyl (*p*NP)-esters were purchased from Fluka (Steinheim, Germany) or from Sigma-Aldrich (USA). Lactic acid, 6-hydroxyhexanoic acid, adipic acid, polycaprolactone (PCL; $M_n = 42500$; $M_w = 65000$), polylactic acid (PLA; $M_n = 30000$; $M_w = 60000$), poly(1,3-propylen adipate) (PPA; $M_n = 42500$; $M_w = 4800$), and poly(R)-3-hydroxybutyrate (PHB) were purchased from Sigma-Aldrich, Fluka or Alfa Aesar (Ward Hill, USA). Restriction endonucleases were from New England Biolabs (Pickering, ON, Canada), T4 DNA ligase was from Roche (Mannheim, Germany) and Titanium DNA polymerase from Clontech (Takara Bio company, USA).

1.2 Protein expression (small scale). A single colony of *E. coli* JM109 cells harboring either YebF-KrCUT or YebF-KrCUT189 was grown at 30 °C on a rotary shaker at 200 rpm in 10 mL Terrific Broth (TB) medium supplemented with 100 µg/ml ampicillin. The overnight culture was used to inoculate 1L fresh TB medium, then grown to OD_{600} 0.4-0.5 under the same culture conditions and induced with 0.1 mM IPTG for 16 h. The cells were collected by centrifugation at 6000 rpm for 30 min (Beckman centrifuge, Model J2-21M) and the supernatant was used as a source of cutinase. Protein concentration was determined using the bicinchoninic acid (BCA) method (Smith et al., 1985).

1.3 Fermentation in DASGIP system (large scale protein expression). A single colony of *E. coli* JM109 cells harboring either YebF-KrCUT or YebF-KrCUT189 were cultured in LB-media containing ampicillin (0.1 g/L) and incubated at 30 °C in a rotary shaker at 200 rpm overnight. The preculture (5% v/v of the total fermentation volume) was added to TB media (1L) in a 2L fermenter (DASGIP, Germany). The temperature was set to 30 °C and the pH was controlled at 7.0 by the addition of concentrated ammonium hydroxide. The cell broth was aerated at 1.0 vvm and stirred at 400-900 rpm to maintain 30% dissolved oxygen. If necessary, the airflow was supplemented with oxygen. At an OD_{600} of 9 (~ 7 h), cutinase expression was induced by the addition of IPTG (final concentration 1mM); the temperature was set to 25 °C, and glucose (33% w/w aqueous solution) was fed at an initial rate of 3 mL/h and

gradually increased to 6 mL/h. The cells were harvested after 16 h and centrifugation of the cell broth (6000 rpm, 4 °C, 20 min) yielded 0.82 to 0.87 L of supernatant that was used to purify the corresponding cutinase.

1.4 Protein purification. All purification procedures were performed at 4 °C on an ÄKTAexplorer™ 100 Air chromatography system (GE Healthcare). Culture supernatant containing active enzyme was concentrated and dialyzed against sodium phosphate buffer (20 mM, pH 7.0) by cross flow filtration using the QuixStand™ system (GE Healthcare) equipped with a hollow fibre cartridge (3 kDa cut-off). The enzyme solution was loaded on a SP-Sepharose™ FF column (XK 50/12) previously equilibrated with 20 mM sodium phosphate buffer (pH 6.0). The flow rate was 4.0 mL/min. The column was washed with the same buffer until no protein could be detected in the flow through, and the enzyme subsequently eluted with a linear gradient of 0 – 0.6 M NaCl. Active fractions were pooled and concentrated by ultrafiltration (membrane exclusion size 10 kDa, in a 100 mL stirring cell (Amicon, USA) and then applied to a HiLoad™ Superdex 75 prep grade (16/60) column previously equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl. Protein was eluted with the same buffer at a flow rate of 1.5 mL/min and collected in 2 mL fractions. The protein profile was monitored by its absorbance at 280 nm.

1.5 Protein sequencing. Purified enzymes were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (BioRAD, USA). N-terminal sequencing was carried out by the Sheldon Biotechnology Centre (McGill University, Montreal, Canada). Phenylthiohydantoin amino acids were analyzed by HPLC using a reversed-phase column.

1.6 Esterase assay. Esterase activity was determined by monitoring the formation of pNP at 410 nm from various pNP-esters using a Beckman UV spectrophotometer (model DU 640) (21). Substrates were dissolved in isopropanol and were added to 50 mM sodium phosphate buffer pH 8.0 to give 10% (v/v) total isopropanol concentration in a 1 mL reaction volume. pNP-butyrate was used at a final concentration of 1.2 mM. For pNP-caprylate, myristate and palmitate, the final concentration ranged from 0.1 to 0.5 mM. The reaction (1 mL) was started by adding an appropriate amount of enzyme. One enzyme unit (U) is defined as the amount of enzyme that produced 1 μmol of product (pNP) per min; specific activity is expressed as U/mg protein. A molar extinction coefficient of 15 mM⁻¹ cm⁻¹ for pNP is used for calculating enzyme activity.

1.7 Determination of cutinolytic activity. Apple (Golden Delicious) cutin was used as substrate. The outer layer of the fruit was peeled by hand and cutin preparation carried out as described by Gerard et al. (1993) with minor modifications. Discs of apple peels were incubated at 50 °C in oxalate buffer [(oxalic acid (4 g/L)/ammonium oxalate (16 g/L)] for 16 h and then collected and washed several times with deionized water. The sample was treated with 0.5 % Novozymes *Aspergillus niger* pectinase (Sigma P2736) in 100 mM phosphate citrate buffer pH 5.8 for 16 h.

The cutin sheets were washed with ethanol, followed by several washes with chloroform, and then incubated in chloroform at 45 °C for 16 h, and finally dried to constant weight. The cutin was ground in a Cyclone sample Mill (UDY Corporation, Fort Collins, Colorado, USA).

A typical reaction mixture (2 mL) contained 20 mg cutin and an appropriate amount of cutinase in 25 mM sodium phosphate buffer, pH 8.0. The mixture was incubated in a water bath at 37 °C with gentle shaking for 16 h. Assays were conducted in duplicate. At the end of the reaction, the remaining cutin was removed by centrifugation (10 min, 5000 rpm). The resulting solution was acidified with hydrochloric acid and cutin monomers extracted with chloroform/methanol, according to Bligh and Dyer (1959). The organic phase was removed by evaporation under an air stream and the dried residue dissolved in 1 mL chloroform/methanol (85:15) and used for further derivatization. Trimethylsilylation of cutin monomers was performed with BSTFA [N, O-bis(trimethylsilyl) trifluoroacetamide] and TMCS (trimethylchlorosilane) provided by the supplier in the ratio 99:1 (Supelco, Bellefonte, USA) at 70 °C for 3 h, and the composition of the mixture determined by GC-MS.

1.8 Analysis of cutin monomers by GC-MS. Cutin monomers were identified by GC-MS on an Agilent 6890 gas chromatography system coupled to a 5973 quadrupole mass spectrometer. 1 µL was injected under splitless condition on a 50 m x 0.20 mm x 0.33 µm DB-5MS capillary column (Agilent). The oven temperature program was 70 °C (for 1.39 min) to 136 °C at 64 °C/min and to 290 °C at 2.5 °C/min (for 55 min). Helium was used as carrier gas. The temperature at the injection port was 250 °C. The detector was run in EI mode at 70 eV. Masses were scanned between 50 and 650 amu.

Components were identified by comparison of the MS spectra of their trimethylsilyl (TMSi) derivatives, as methyl ester TMSi ether or as TMSi ester TMSi ether, with published spectra (Kallio et al., 2006; Philipps et al., 2002).

1.9 HPLC-analysis of monomers after polymer degradation. HPLC analysis was carried out on a Waters system consisting of a model 600 pump and 717 Plus autosampler, equipped with a refractive index detector (Waters, model 2414) or PDA detector (model 2996). Organic acids were analyzed with a Transgenomic ICsep IC-ION-300 (300 mm x 7.8 mm OD) column using 0.01N H₂SO₄ as mobile phase at a flow rate of 0.4 mL/min at 35 °C. Retention times are as follows: lactic acid, 23.2 min; 6-hydroxyhexanoic acid, 45.2 min; and adipic acid, 33.4 min.

1.10 Determination of polymer degradation by size exclusion chromatography. Size exclusion chromatography (SEC) was performed using a multi-detection system from Viscotek (Houston, TX) consisting of a Model 302 Triple Detector Platform, including a refractive index detector, a four capillary viscometer and a 2 angles laser light scattering detector, and a GPCmax Integrated pump, autosampler, and degasser. Molar masses of the samples were determined with universal calibration with the refractive index detector and the viscosity detector. The

universal calibration curve was based on polystyrene standards [Polymer Laboratories (Amherst, MA) and Aldrich (Oakville, ON)] using the molar masses determined by the manufacturer and the intrinsic viscosities measured by the apparatus. The software OmniSEC™ software from Viscotek was used for data collection and calibration. Separation in THF was performed by injecting 100 µL of 1.5-2 mg/mL solutions of standards or samples into thermostatically controlled SuperRes™ columns [35 °C; PAS-102, PAS102.5, PAS-103L, each 300 mm x 8 mm; PolyAnalytik (London, ON)]. The flow rate was 1 mL/min.

1.11 Degradation of polyesters. An appropriate amount of cutinase was added to 20 mg of the polymer to be analyzed (PCL, PLA, PHB, and PPA) in 50 mM phosphate buffer, pH 8. The reaction mixture (2 mL) was shaken at 37 °C at 175 rpm on an orbital shaker. Samples were taken after 1, 3, 6, and 24 h and aliquots of 50 µL were analyzed by HPLC; the remaining mixtures were frozen and subsequently lyophilised. The resulting solid (~ 40–50 mg) was dissolved in THF, filtered, diluted (1.5-2 mg/mL), and analyzed by SEC.

Alternatively, a thin film was prepared from 1 g of commercial available PCL pellets and used for degradation. The polymer was dissolved in dichloromethane in a 1 L beaker. The solvent was allowed to evaporate overnight and the resulting film dried to constant weight under reduced pressure. Prior to enzymatic treatment the film was cut into pieces (20 mg) of approximately 1.5 cm x 1.5 cm and thickness of approximately 0.1 mm. The reaction mixture was monitored by HPLC and SEC as described above.

1.12 Action of KrCUT on plant material. Hemp stems (50 cm, 25 g) were treated enzymatically with KrCUT (100 U/g) together with a cloned pectate lyase (20 U/g) described by Xiao et al. (2008). The reaction was carried out in 50 mM Tris-HCl, pH 8.0 supplemented with 0.5 mM CaCl₂ (required for pectate lyase). The samples were incubated at 37 °C with gentle shaking at 100 rpm for 16 h. The stems were removed from the enzymatic solution, peeled off by hand, and the resulting fibre washed with hot water to inactivate the enzymes. After removal of the outer layer debris by rinsing with cold water, the fibres were air-dried. A sample of the hemp stem treated only with pectate lyase was prepared for comparison. These experiments were conducted in duplicate. The dried fibres were spread onto a microscope slide and observed by light microscope. A scanning electron microscopy (SEM) analysis was also conducted using a Hitachi S-4700 at a voltage of 2 kV to observe the fibre surface and also fiber bundle.

1.13 Effect of KrCUT in removing pectin from hemp fibre. Hemp fibres (German variety, 150 mg) were treated with either pectate lyase (PL, 10 U) or PL combined with various amounts of KrCUT (40, 60 and 100 U). Control experiments containing no enzyme or 100 U of KrCUT were conducted for comparison. After incubation at 37 °C, aliquots of the reaction mixture were taken at 1 h 30 min and 3 h, and analyzed for pectin degradation products using the thio-barbituric acid (TBA) method (52) which detects released unsaturated compounds spectrophotometrically at 550 nm.

1.14 Adsorption assays. Adsorption assays were carried out following procedures of Kasuya et al. (1999). Purified KrCUT and KrCUT189 at concentrations ranging from 0.16 to 3.2 mg/mL were added to a suspension of 25 mg PHB in 50 mM sodium phosphate (pH 8.0) in a total volume 1 mL. The mixture was incubated at room temperature with gentle shaking for at least 3 h. The PHB polymer was removed by centrifugation and the concentration of protein in the supernatant was determined by the BCA method. The concentration of bound protein at a particular concentration of cutinase was calculated as the difference between a control without added PHB and free cutinase after incubation with PHB. An isotherm of [Bound] (mg/mg PHB) vs [Free] (mM) was generated and binding parameters were determined by non-linear regression using the Langmuir equation:

$$E_{ad} = E_{max} \left(\frac{K[E]e}{1 + K[E]e} \right)$$

where $[E] = E_{ad} + [E]e$, is the concentration of the protein added; E_{max} is the maximum amount of protein adsorbed on the polyester granules, and K is the adsorption equilibrium constant of the protein.

Example 2:

Sequence and phylogenetic analyses of cutinase from *K. radiotolerans*

The 885-bp Krad_4111 locus tag of the *K. radiotolerans* genome (YP_001363838.1; Copeland, 2007) was selected as the result of a BLAST search using the amino acid sequence of FsCUT as a query sequence. A polypeptide having 294 amino acids corresponding to the full length sequence of a cutinase from *K. radiotolerans* was identified. A bioinformatic analysis of the 294-amino acid sequence of full length KrCUT (hereinafter referred to as "**ssKrCUT**"; **SEQ ID NO: 1**) revealed a modular domain organization consisting of a 33-amino-acid signal peptide (amino acids 1-33 of SEQ ID NO: 1), a catalytic domain (amino acids 34-222 of SEQ ID NO: 1; **SEQ ID NO: 2**), and unlike other characterized cutinases, the presence of a C-terminal extension made up of a Pro/Thr-rich linker (amino acids 223-239 of SEQ ID NO: 1; **SEQ ID NO: 3**), and a designated polymer-binding domain (amino acids 240-294 of SEQ ID NO: 1; **SEQ ID NO: 4**) (Fig. 1).

Example 3:

Cloning of cutinase-encoding gene and variants

A synthetic gene encoding full-length cutinase from *K. radiotolerans* identified in Example 2 but lacking the 33-amino acid endogenous N-terminal signal sequence was constructed after codon optimization by the reduction of G+C content from 77.2% to 59% for its expression in *E. coli*. The synthetic gene having the nucleotide sequence set forth in **SEQ ID NO: 5** was cloned in a recombinant pUC57 plasmid provided by GenScript Corporation (Piscataway, NJ USA). The specific primers, P1: 5'-GAGCTGCAAGCGACTTGCACTGACGTC-3' and P2 5'-GCAGAAGCTTTCAAC AAGCAGTTACCAAG-3', were then designed to amplify the mature portion of the KrCUT-

encoding gene (amino acids 34-294 of SEQ ID NO: 1; **SEQ ID NO: 6**; herein referred to as "KrCUT"); P1 and P3: 5'-TGTAAGCTTTCATGTGGTAGGA ACCGGCGT-3' were used to amplify a truncated version KrCUT206 (amino acids 34-222 of SEQ ID NO: 1; **SEQ ID NO: 2**; herein referred to as "KrCUT189") lacking the C-terminal 72 amino acids, which includes the T/P-rich linker as well as the polymer-binding domain. The indicated primer sets were designed to incorporate a 5' PstI site and 3' HindIII site (underlined) for directional cloning in the IPTG-inducible vector pKK223-3. The PCR parameters were: 94 °C 3 min, 30 cycles of 94 °C for 45 s, Tm-3 °C for 30 s, 68 °C for 1 min, followed by a final extension at 68 °C for 10 min.

To express KrCUT and KrCUT189 in a secreted form, the *E. coli* extracellular carrier protein YebF (Zhang et al., 2006; **SEQ ID NO: 8**) was used to construct fusion proteins. The gene encoding YebF (366-bp minus stop codon but including the signal peptide; **SEQ ID NO: 7**) was amplified by oligonucleotides P4: 5'-CGAGGAATTCATGGAGAAAAACATGAAAAAAG-3' and P5: 5'-GAACTGCAGACGCCGCTGATATTCGCGC-3', containing the EcoRI and PstI restriction sites (underlined). The PCR fragment was gel purified and cloned upstream of the cutinase gene in pKK223-2 to produce pKK223-3-YebF-KrCUT and pKK223-3-YebF-KrCUT189. These recombinant plasmids were transformed into *E. coli* JM109 cells by conventional techniques (Sambrook et al., 1989). The cloned genes were verified by DNA sequencing using a Big Dye™ DNA sequencing kit (Applied Biosystems) and an automated DNA sequencer (Model 377, ABI Prism). The nucleotide sequence encoding the YebF-KrCUT fusion protein is set forth in **SEQ ID NO: 9** and the corresponding amino acid sequence of YebF-KrCUT is set forth in **SEQ ID NO: 10**. The nucleotide sequence encoding the YebF-KrCUT189 fusion protein is set forth in **SEQ ID NO: 11** and the corresponding amino acid sequence of YebF-KrCUT189 is set forth in **SEQ ID NO: 12**.

Example 4:

Expression and purification of YebF-KrCUT and YebF-KrCUT189 fusion proteins

The YebF-KrCUT and YebF-KrCUT189 fusion proteins were expressed from *E. coli* as described in **Example 1**, sections 1.2 and 1.3.

Cutinase activity, measured using pNP-butyrate as substrate as described in **Example 1**, section 1.6, was detected in *E. coli* [pKK233-2-YebF-KrCUT and pKK233-2-YebF-KrCUT189] culture 16 h after IPTG induction with the maximum activity found by 20 h (not shown).

Production of the 39-kDa YebF-KrCUT (**Fig. 2A, lane 1**) fusion protein was confirmed by SDS-PAGE analysis), consistent with the summed masses of the 10-kDa YebF carrier protein and the 29-kDa mature KrCUT. **Fig. 2A, lane 1**, resulted from one column purification step using the SP-Sepharose™ FF as described in **Example 1**, section 1.4. Production of the YebF-KrCUT189 fusion protein was also confirmed by SDS-PAGE analysis.

The YebF-KrCUT fusion protein was further purified as described in **Example 1**, section 1.4 to electrophoretic homogeneity in two steps involving cation exchange and size exclusion chromatography. Proteins bound on the SP-Sepharose™ eluted in two active peaks: peak 1 (minor) at a lower ionic strength, and peak 2

(major) at higher ionic strength (not shown). The height of peak 1 eluting from the SP-Sepharose™ column increased over time while peak 2 decreased in height, indicating some unknown processing of peak 1 protein species under storage conditions (not shown). This was confirmed by SDS-PAGE analysis of fractions from the major peak 2, which showed two protein bands of M_r 39-kDa and 31-kDa (for YebF-KrCUT). These two species could be separated by an additional SEC step (**Fig. 2, lanes 2 and 3**). In **Fig. 2A, lanes 2 and 3**, the two bands from lane 1 were separated by loading the fraction of lane 1 on a HiLoad Superdex™ 75 prep grade (16/60) column.

The YebF-KrCUT189 fusion protein, which also eluted in two active peaks, was purified to electrophoretic homogeneity the same way as for YebF-KrCUT. **Fig. 2B, lane 4** represents YebF-KrCUT189 from HiLoad™ Superdex™ 75 prep grade (16/60) column, the fraction was collected after a first column using SP-Sepharose FF.

N-terminal amino acid sequencing performed as described in **Example 1, section 1.5** helped to establish the identity of each of the protein bands with respect to YebF-KrCUT and YebF-KrCUT189. The procedure is described with respect to YebF-KrCUT in the following paragraph, but a similar approach was used to establish the identity of each of the protein bands for YebF-KrCUT189.

The sequence ANNETSKSVT derived from the 39 kDa protein band corresponds to the first 10 amino acids of the mature portion of YebF, confirming previous result that the carrier protein is cleaved immediately after the 21-amino acid secretory leader sequence (Zhang et al., 2006). The N-terminal amino acid sequence (AT[A]SDVDVVF) of the 31-kDa species is virtually identical to the first ten amino acids of the mature cutinase. The discrepancy of Ala in the third position versus Cys in the DNA-predicted sequence is due to the fact that Cys without modification is not detectable by Edman degradation.

The above analysis confirmed that when the YebF-KrCUT and YebF-KrCUT189 proteins were purified immediately after fermentation, they exclusively yielded the intact YebF-KrCUT and YebF-KrCUT189 fusion proteins, respectively. Upon storage for several weeks at 4°C, the intact fusion proteins underwent processing to produce a lower molecular weight species corresponding to the cleavage of the YebF portion, resulting in the polypeptides KrCUT and KrCUT189.

The presence of YebF appeared to greatly decrease the activity of the fusion proteins. For example, the processed KrCUT exhibited some three-time higher specific activity compared to that of YebF-KrCUT (162 versus 47 U/mg with *p*NP-palmitate as substrate). The results are shown in **Fig. 6**, in which fractions C1-C10 correspond to the first peak (KrCUT) and fractions D9-D11 correspond to the second peak (YebF-KrCUT).

Example 5:

Substrate range

The KrCUT189 protein was purified in a similar way as YebF-KrCUT as described above (**Fig. 2B**) and its activity towards a number of *p*-NP esters was compared to those of KrCUT (**Fig. 3**). With KrCUT, the highest activity was obtained with *p*NP butyrate showing a 8-fold higher activity over *p*NP-palmitate which is in accordance with the

activity profile of other cutinases (Kim et al., 2003). The KrCUT189 variant generally gave lower activity with various substrates compared to the full length protein. The highest activity of KrCUT189 was towards pNP-myristate, which was surprisingly higher than that of KrCUT. In both cases, there is little activity towards pNB-acetate.

5

Example 6:

pH optimum and thermostability

Both KrCUT and KrCUT189 showed a pH optimum of 8 (Fig. 4). Universal Buffer solutions were prepared according to Britton and Robinson (Surinenaite et al., 2002), having the selected pH value from 5-11. Activity of KrCUT and KrCUT189 were measured at the indicated pH, using p-nitrophenyl butyrate as substrate as described in

10 **Example 1, section 1.6**). Curves were obtained by plotting values of activity against pH.

Enzyme stability was studied over the temperature range of 40-60°C using purified KrCUT. Aliquots were withdrawn at defined times (10, 30 and 80 min), chilled on ice, and assayed for activity against p-NP butyrate at room temperature. KrCUT appeared thermostable up to 45°C. At 50°C and above there was virtually no activity left after 10 min of heat treatment (Fig. 5).

15

Example 7:

Potential inhibitors of cutinase activity

KrCUT cutinase activity was assayed as described in **Example 1, section 1.6** in the presence of various chemicals using p-NP palmitate as substrate under standard assay conditions. The zinc ion dramatically inhibited

20 cutinase activity with a complete loss of activity at 1 mM and over 90% at 0.1 mM. Mg²⁺ showed only 19% enzyme inhibition at 10 mM; and 50% for Fe²⁺ at 1 mM. Cu²⁺ at 1.0 mM had little effect. The enzyme was not affected by the metal-chelating agent EDTA even at 10 mM. The anionic surfactant SDS was a strong inhibitor of cutinase even at 0.01 % retaining only 28% activity. 0.1% SDS resulted in a complete loss of activity. Organic solvents, e.g. alcohols exhibited a strong inhibiting effect on cutinase in concentration above 15-20%. In particular, the 2-propanol used to

25 dissolve the substrate and that is present in the standard assay at 10 % (v/v), strongly reduces enzyme activity when it exceeds 15%. Dimethylsulfoxide (at 30%) and dimethylformide (at 5%) inhibited cutinase activity by about 70%. Interestingly, *para*-chloromercuribenzoate at concentrations 0.01 to 1mM had no discernible effect on KrCUT implying that there is no free sulfhydryl group among the six cysteines found in KrCUT.

30

Example 8:

Hydrolysis of cutin

To measure cutinase activity, assays of cutin hydrolysis were performed as described in **Example 1, section 1.7** at two different enzyme concentrations. Characteristic cutin components, C₁₆ and C₁₈ hydroxyl fatty acids, were identified by GC-MS analysis. One representative mass spectrum of the enzymatic reaction is shown in

Fig. 7. 18-Hydroxy-octadeca-9,12-dienoic acid, 10,16-dihydroxyhexadecanoic acid and 9,10,18-trihydroxyoctadecanoic acid, with retention times of 55.13, 55.92 and 64.20, respectively, were found to be the major products of hydrolysis (**Table 1**).

Table 1:

5 **Momomeric products released from apple cutin by cloned *K. radiotolerans* cutinase.** Hydrolysis was carried out at two different units of activity of cutinase. Not detected (n.d)

Retention time	Cutin hydrolysis products	20 Units Area (%)	200 Units Area (%)
55.13	18-hydroxy-octadeca-9,12-dienoic acid	18.0 ± 6.3	2.22 ± 0.13
55.24	9,16-dihydroxyhexadecanoic acid	n.d	3.81 ± 0.62
55.92	10,16-dihydroxyhexadecanoic acid	39.25 ± 2.5	27.74 ± 0.41
63.37	9,10,18-trihydroxyoctadec-10,12-dienoic acid	n.d	13.25 ± 0.40
64.20	9,10,18-trihydroxyoctadecanoic acid	n.d	25.55 ± 0.10
66.19	9,10,18-trihydroxyoctadec-9 enoic acid	n.d	6.25 ± 0.40

Additional components of C16 and C18 fatty acids were identified as 9,16-dihydroxyhexadecanoic acid (55.24 min), 9,10,18-trihydroxyoctadec-10,12-dienoic acid (63.37 min), and 9,10,18-trihydroxyoctadec-9 enoic acid
10 (66.19 min).

Example 9:

Degradation of synthetic polyesters by KrCUT and KrCut189

An initial plate clearing assay indicated that a lawn of *E. coli* JM109 cells harboring YebF-KrCUT was
15 capable of producing a large clearing zone with PCL as substrate (not shown). To quantify this further, pellets of polycaprolactone (PCL) as well as films of PCL were incubated with KrCUT at various enzyme concentrations (1.25 μM, 2.5 μM, and 5 μM representing 4.7, 9.5 and 19 enzyme Units) at 37 °C. The degradation of the polymer was followed over time by analyzing aliquots of the reaction mixture by HPLC for the detection of the released monomer (6-hydroxyhexanoic acid) and characterization of the remaining polymer by SEC after lypholization of the sample.
20 KrCUT was able to release nearly 90% of the acidic monomers with enzyme concentration of either 2.5 or 5 μM in 6 hours. Even with a very low concentration of the enzyme (1.25 μM) thin films of PCL were nearly completely degraded after 24 hours (**Fig. 8A**).

SEC analysis confirmed the results obtained by HPLC. Comparison of the peak area corresponding to that of the polymer showed a gradual decrease over time with complete disappearance of the polymer in experiments
25 conducted with 2.5 - 5 μM after 24 hours. This correlated well with the increase in acid detected (**Table 2**).

Table 2

Evidence of PCL degradation: comparison of SEC and HPLC analysis

time (h)	PCL (SEC)	M _n (Daltons)	M _w (Daltons)	acid (HPLC)
0	100%	44000	63300	0%
1	61%	53800	64400	28%
3	27%	55000	66600	60%
6	12%	58200	68100	85%
24	0.3%	69500	74000	100%

A dramatic increase in M_n and M_w from 44000 and 63300 to 69500 and 74000 emphasized the preference of KrCUT for low molecular polymers. The degradation of PCL pellets proceeded more slowly with 18% of the maximum yield of 6-hydroxy hexanoic acid were detected in the reaction mixture after incubation with 2.5 μM of protein for 24 hours (Fig. 8A). The decrease in depolymerization is presumably due to the limited surface area of the PCL pellets compared to the films. Nearly identical results were obtained when PCL pellets and PCL films were incubated with KrCUT189 (2.5 μM) (Fig. 8A).

Both KrCUT and KrCUT189 (2.5 μM; 2 Units) only degraded approximately 5% of PLA (Fig. 8B). However, in the case of poly(1,3-propylene adipate), a polymer consisting of alternating diacid and diol subunits, 12% and 17% of adipic acid were found in the reaction mixture containing KrCUT and KrCUT189, respectively, after 24 hours (Fig. 8B). All in all, it appears that both enzymes exhibited a preference for longer chain monomers such as 6-hydroxy hexanoic acid and adipic acid (both C6 unit in polymer) compared to lactic acid (C2 unit).

Example 10:

Polymer-binding domain of KrCUT

Different polymers (cellulose, PLA, PCL, and PHB) were tested to evaluate the ability of KrCUT and KrCUT189 to bind these materials. Binding assays for PCL and PLA could not be performed since both polymers were degraded by the enzymes. KrCUT did not bind to cellulose, indicating that the binding domain of the cutinase is distinct from a cellulose binding domain (CBD). On the other hand, KrCUT binds efficiently to PHB but did not degrade. The KrCUT189 variant showed no binding to PHB, indicating that essentiality of the C-terminal extension for this activity.

The kinetics of KrCUT adsorption to PHB is shown in Fig. 9. The curve shows the relation between the adsorbed cutinase and the equilibrium concentration of the enzyme, with the adsorption to the PHB surface expressed by the Langmuir adsorption equation. As a result, the maximum amount of cutinase absorbed on the PHB granules (E_{max}) was determined to be 23.68 ± 0.03 μg/mg, and the adsorption equilibrium constant (K) was calculated to be 0.12 ± 0.03 ml/mg PHB.

Example 11:**Effect of KrCUT on hemp fibre**

The quantity of pectin released from natural hemp fibre by KrCUT in conjunction with the action of a thermostable pectate lyase was assessed as described in **Example 1, section 1.13**. The thermostable pectate lyase alone released a certain amount of pectin as expected of the pectinolytic activity (Xiao et al., 2008). With an increasing amount of added cutinase, a higher percentage of pectin degradation products were detected when the quantity of added pectate lyase was fixed. When KrCUT was incubated with hemp fibre at the highest concentration in the absence of pectate lyase, negligible amounts of pectin from the fibre material were detected as compared to a control reaction without any added enzyme (**Fig. 10**).

The effect of KrCUT on the hemp fibre integrity was visualized using light microscopy and SEM. Light microscopic images revealed that treatment of fibres by both cutinase and pectate lyase appeared to produce more separated fibre bundles, whereas pectate lyase alone showed more fibres embedded in pectin and wax material (**Fig. 11**). The SEM pictures showed that cutinase treatment gave more separated fibres having a cleaner surface, compared to those of pectate lyase treated samples.

Example 12:**Effect of KrCUT on inactivation of mycotoxin**

Fungi strains *Aspergillus flavus* and *Aspergillus niger* were cultivated and used for Aflatoxin and Ochratoxin isolation, respectively.

Reactions were performed in 2 mL volumes in Eppendorf tubes comprising the mycotoxin to be analyzed and 0.3 mg/mL KrCUT enzyme. Ochratoxin A was added at a concentration of 200 ppb. The samples for Aflatoxin contain the B1 and B2 types of the mycotoxin in the concentration of 600 ppb and 9 ppb, respectively. In control reactions, the enzyme volume was replaced by an equivalent amount of water. The reactions were incubated at 37 °C, samples were withdrawn at time periods 0, 1 h, 2 h, followed by the addition of equal volume of methanol 80% (v/v) for extraction. Reactions were stored, if needed until chromatographic analysis.

Analysis: samples were centrifugated and the supernatant analyzed for Ochratoxin A and Aflatoxin by LC-MS/MS as described by Rudrabhatla et al., 2007. Results are shown in **Tables 3 and 4**.

Table 3:
Effect of KrCUT on Ochratoxin

Incubation time (h)	Residual Ochratoxin %
0	100
1	56.63
2	0.68

Table 4:
Effect of KrCUT on Aflatoxin mixture

Incubation time (h)	Residual Aflatoxin B1 (%)	Residual Aflatoxin B2 (%)
0	100	100
3	48	48

The results in **Tables 3 and 4** show that the KrCUT is able to act on both aflatoxin and ochratoxin, in contrast to most other cutinases that can act only on one type of mycotoxin.

Example 13:
Effect of KrCUT on inactivation of mycotoxin

Fungi strains *Aspergillus flavus* and *Aspergillus niger* were cultivated and used for Aflatoxin and Ochratoxin isolation, respectively.

Reactions were performed in 2 mL volumes in Eppendorf tubes comprising the mycotoxin to be analyzed and 0.3 mg/mL enzyme. The samples for Aflatoxin contain the B1 type of the mycotoxin in the concentration of 1000 ppb. In control reactions, the enzyme volume was replaced by an equivalent amount of water. The reactions were incubated at 37 °C, samples were withdrawn at time periods 0, 1 h, 2 h, 4 h and 24 h followed by the addition of equal volume of methanol 80% (v/v) for extraction. Reactions were stored, if needed until chromatographic analysis.

Analysis: samples were centrifugated and the supernatant analysed for Ochratoxin A and Aflatoxin by LC-MS/MS as described by Rudrabhatla et al., 2007. Results are shown in **Table 5**.

Table 5:
Effect of KrCUT on Aflatoxin

Incubation time (h)	Residual Aflatoxin B1 (%)
0	100
1	80
2	61.5
4	49
24	20

The results in **Table 5** show that KrCUT is able to reduce amount of 1000 ppb Aflatoxin B1 to 200 ppb within 24 h.

Example 14:

Construction of recombinant fusion polypeptides having a cutinase catalytic domain operably linked to a polymer-binding domain

5 Recombinant fusion polypeptides having a cutinase catalytic domain operably linked to a polymer-binding domain may be constructed using basic recombinant DNA cloning techniques, making use of suitable domains (cutinase catalytic domains, linker domains, and polymer binding-domains) from different cutinases. Examples of different cutinases and the domains therein are shown in Table 6.

Table 6. Examples of different cutinases and the domains therein

Gene/protein name / organism	Cutinase amino acid sequence (signal sequences are in lower case letters; cutinase catalytic domains are in bold ; linkers are highlighted in black ; polymer binding domains are <u>underlined</u>)	SEQ ID NO:
>GI:502883354 WP_013118330 cutinase [<i>Cellulomonas flavigena</i>]	mqvtptrrtlagvvaavaalasvttlatgasaa APACPDVELVFARGTG EAAAGLGI VGRPLERALAAELPGRTVVATAVDYAASSSQASAGPGSGDMVAKVRSRAAACPGT QFVLGGYSQGATVTDLALGIRTGVTAGTALPEDLAARVAAVVVYGNPLGLTRRTI AQAAPAFATRTVEYCNAGDPVCEPGGGRFTAHTYATNGTVLEGARFAAARVTAP APTPTPGPTGTPAPVPSAEP TAPGDT CVTASSLQHVRDGRAYPLWMRTYARGSG DPLGVLSSRTVVSLQADGTDTRKVAAC	13
>gi 697974256 gb K GM14009.1 cutinase [<i>Cellulomonas bogoriensis</i> 69B4 = DSM 16987]	msitpggrkvaaalittaaltstaaiaatpataaapvaggpvatsTGDCPDVHVFA RGTGEPRGLGIVGRPFVSDLGDALPTMTVTSYAVNYSANASQTSAGPGAGDMTSH VTSMAARCPGTQFVLGGYSQGATVTSIAGVARSTSIRSRVLPANLEPRVAAVVVF GNPLGLTRRTIASEAPAYAAKSRDYCNRSDTVCGGRGDARGGHLAYVSNQSVADG AAFAASLVGVAPGDPAS CVTARAVDHVEADRAFRSVLRAYARGSRDPLGRLTSSD LVSLQRTGQDSWSVPAC	14
cutinase [<i>Cellulomonas bogoriensis</i> 69B4 = DSM 16987]; accession KGM14008.1	mstspvrtrltlaallaaglaatsvalspaasatslpaaaplsAGSCPDVQVVFAR GTGERAGLGIIGRPFARALADELPGMTVTSHAVDYAAAAAQRSAGPGATAMTDHV TAMAARCPGTQFVLGGYSQGATVTSIALGIRAGTTTGRAIPDELSDRVAAVVVF NPLGMRGQTIASASRTYADRAKDYCNQSGDSICGRQPSTGRGTHGTGYATNGSTTDG ARFAAGLVTANPPEAAPPTAPPTPTPTAPP SDQVTCVTAQVSEHVEARRAIRG LTRAYARGTLEDIGRLRSTEEVSLRRSGTFSWTPTASC	25
>gi 697886498 gb K GM0336.1 hypothetical protein Q760_05675 [<i>Cellulomonas cellasea</i> DSM 20118]	mhqltppaapsrpraarrprllaaalalaatsgalstlaaptava APACADVEV LFARGTGEPGLGLVLTGTFVRSVTSALSDRTVTSYAVNYAAESSQRSAGPGATDL TNHLTATAAACPGTRFVLGGYSQGATVVDLALGIRTGTTTGTAIPAALPRVA VVFGNPLGISGRTIATASPTYAARARDFCATGDPVCGGGSSFAAHLAYRTNGDVT AGADFAA GLARATTVPVPTPTVP GTPTPSPTAPGTPTPVPTTSPTPAPSPTAPGA ACVRASTRAHVEAGRAERRHGIAYATGSGDRIGWVSSFVRVSVQQTADGWERVLS C	15
>gi 521090090 ref WP_020420995.1 cutinase [<i>Amycolatopsis</i> sp. ATCC 39116]	mvhnrlrkltlacavvlagtatvgtasgatsaa ACSDLEIVFARGSGEAPGLG ITGTPLVSDVKSALSSASVSSYAVDYAASYDQTSAGPGATDMSNHKSTAAACPD TKFAIGGYSQGASVTDIAIGIRTYLGTGQTIPTTELAPRVAVIAFGNPLGLYGQT IKTASPTYGPKSLEFCNRGDNVCGGTGTGPGYGHLGYARDGSVDQAAAFIAKQYN AS	16
>gi 648619273.1- 219 cutinase [<i>Frankia</i> sp. BMG5.12]	mrlarswkqgravavfltavagtlgagitaspvs AATCSDVDVVFARGSGELPGLG ITGTPFVNSVKQGLTGKTVSSYAVNYAADIAQTSAGATDMTRHVRVVAASCPN TKFVLGGYSQGASVTDISIGIRTFLGSGETIPTTELAPRVAAVVVFVGNPLALFGQK ITTASPLYGPKAKEFCNLGDPVCAGGFNVFAHLTYGFDGSTANGASFVSKVRA	17
>gi 383779171 ref Y P_005463737.1 putative cutinase [<i>Actinoplanes missouriensis</i> 431]	mnkrrkrnvatvgicaavvaagavitpqafagtlfggrattaAGDCSDVELVFAR GTGEPQGLGIVGRPLARELAAALPDLAGVSFAVVYAAAGNQRSAGPGATNMSRHI TEVAGECPDTRFVIGGYSQGASVTDIAIGIRGAGTAGEAIPERLADRVAAVVVF NPLGLQRRTIAGSSAVFGPKAKEFCNTGDPVCGGGGNFAAHLAYPRNGSVQAAA FAASKIAG	18

>gi 589395439 gb E XG80196.1 Cutinase [Cryptosporangium arvum DSM 44712]	msvlltvrrrvvtavaltatgltgiafaaspaTAACSDVQVVFARGSTEAPGLGIL GRPLVSAVQQQLPGLTVDSYAVDYAANVSQTSAGPGATDMSDHITEVAARCPDTE FVIGGYSQGASVTDIAIGIRTTLGRGGTIPENLAPRIKAVTVFGNPLRLSRQTIN SASQLYGRKAIDICATGDPVCGNGANAAHLRYAFDGSVTRAAQFAANLVRTT	19
>gi 653334177 ref WP_027494901.1 MULTISPECIES: cutinase [Rhodococcus]	mkrarliaysiaalaisatavalptgvasAAPCSDVDVVSFARGTGELPGLGITGTP FVNSVKSQLSDRSVSTYAVNYAADFTQASAGPGSRDLVAHLNSVAASCPSTKFVI GGYSQGATVVTNAVGLRTPSSFTGAVIPAAIADRIEAVVVFGNPFGLTGRKIETA SSTYGSRNSFCNFGDPVCQIGGFNTFAHLTYGTNGSTTQGASFQAAQVRS	20

Further examples of constructions of fusion proteins are shown below.

14.1 Fusion protein CfKr

5 Amino acid sequences of this construct contain the catalytic domain of cutinase from *Cellulomonas flavigena* (CfCUT) fused to the C-terminal region (T/P-rich linker and polymer binding domain) from *Kinecoccus radiotolerans* cutinase. The amino acid sequence of this fusion protein is set forth in **SEQ ID NO: 21**, in which residues 1-187 correspond to the cutinase catalytic domain from CfCUT, residues 188-206 correspond to the T/P-rich linker of KrCUT, and residues 207-262 correspond to the polymer binding domain of KrCUT. To ensure extracellular
10 secretion of this fusion protein, the N-terminus thereof can be fused to the C-terminus of YebF (**SEQ ID NO: 8**) to give YebF-CfKr encoded by the nucleotide sequence of **SEQ ID NO: 22**.

14.2 Fusion protein KrCf

Amino acid sequences of this construct contain the cutinase catalytic domain of KrCUT (**SEQ ID NO: 2**)
15 fused to the T/P-rich linker of KrCUT (**SEQ ID NO: 3**), which in turn is fused to polymer binding domain of CfCUT. The amino acid sequence of this fusion protein is set forth in **SEQ ID NO: 23**, in which residues 1-189 correspond to the cutinase catalytic domain from KrCUT, residues 190-206 correspond to the T/P-rich linker of KrCUT, and residues 207-262 correspond to the polymer binding domain of CfCUT. To ensure extracellular secretion of this fusion protein, the N-terminus thereof can be fused to the C-terminus of YebF (**SEQ ID NO: 8**) to give YebF-KrCf
20 encoded by the nucleotide sequence of **SEQ ID NO: 24**.

Example 15:

Long term stability of KrCUT in culture medium

25 Stability over time and/or with temperature represents one of the main desirable properties of an enzyme, for their utilization as ingredients in cleaning product formulation. Most enzymes tend to lose activity with the time.

The KrCUT was routinely produced in the form of secreted fusion protein, YebF-KrCUT in culture medium. Culture medium having KrCUT activity was stored at 4°C without any additives. Activity was determined periodically by using p-nitrophenyl laurate as substrate, and reported in **Table 7**.

Table 7. Storage stability of KrCUT in culture medium at 4°C

Months	0	3	4	17	19
Activity (U/mL)	3.0 ± 0.94	25.67 ± 0.94	17.13 ± 0.70	16.66 ± 2.47	21.94 ± 1.83

At room temperature, KrCUT in culture medium without additives retains full activity for a period of 4 months.

Example 16:

Stability of KrCUT in stabilisation buffer

For stability testing, culture medium showing KrCUT activity, and containing 100 mg total protein was used. Stabilisation buffer composed of sodium gluconate 1-5%, sodium carbonate 1-5%, sodium citrate 1-5% and propylene glycol 1-5 % was prepared; 50 mL enzyme solution was added to 200 mL of buffer and stored at room temperature, after mixing. Samples were taken at defined time periods and the enzymatic activity was measured using p-nitrophenyl dodecanoate as substrate.

Activity loss could be observed only after 4 months, and up to 65 % of the initial activity was present after 9 months at room temperature.

Data are reported in **Figure 12**.

For most of enzymes, more complex mixtures with different ingredients at higher concentrations are used for stabilisation, namely polyol such as propylene glycol and glycerol, boric acid and boron derivatives and sodium formate.

In some test cleaning product formulations, the KrCUT enzyme was combined with a mix of proteases from *Bacillus*. The efficacy of the cleaning product was reduced, demonstrating the contribution of the KrCUT to the overall efficacy of the cleaning product. However, the loss of KrCUT activity could be avoided or substantially reduced by the additional of stabilizers to the formulation (e.g., polyols and/or boric acid).

Example 17:

Stability of KrCUT in the presence of metal ions

Enzymes such as lipases, amylases, and proteases are widely used in cleaning product formulations, and these types of enzymes are generally sensitive to the presence or absence of metal ions. For example, lipases generally require calcium for optimal activity. Amylases and proteases also generally require metal ions for optimal activity.

For improved performance, chelating agents (namely EDTA) are often added to cleaning products. These chelating agents sequester metal ions and thus may negatively impact enzyme activity (e.g. of lipases, amylases, and proteases).

Interestingly, activity of KrCUT is not affected by EDTA or other chelating agents often found in cleaning products. More specifically, 10% w/v of EDTA does not inhibit KrCUT activity (data not shown), and the enzyme retains 100% activity in the presence of GLDA (L-Glutamic acid N,N-Diacetic acid Tetra sodium), at a concentration of 5% w/v.

More particularly, stability in the presence of hydrogen peroxide makes KrCUT an interesting candidate for cleaning products with disinfection properties.

Example 18:

Construction and expression of recombinant enzymes having cutinase activity

The recombinant enzymes listed in Table 8 were constructed, expressed and purified for further characterization as generally described in Examples 3 and 4.

Table 8. Recombinant enzymes having cutinase activity

Enzyme	Organism(s)	Amino acid sequence (YebF carrier proteins are in lower case letters; cutinase catalytic domains are in bold; linkers are highlighted in black; polymer-binding domains are underlined)	SEQ ID NO: (without YebF)
YebF-KrCUT	<i>Kineococcus radiotolerans</i>	meknmkkrqafllglllvsacasvfaannetsksvtfpkcedldaagiaas vkrdyqqnrvarwaddqkivgqadpvawvslqdiqgkddkwsvpltvrgk sadihyqvsdckagmaeyqrrATCSDVDVVFARGTGETPGLGVVGGPFV RSLTGELSDRTVTSHAVDYAASSSQASAGPGATAMSAHVREVAAACPSTR FVLGGYSQGATVTDIALGIRTGTTTGTTPVPAELAGRVAADVVFNGNPLGLS GRTIATASSTYGPCKSKDYCNSSDSVCGSAPKTGTGGHLSYASNGSTTDGA RFAAGLVRAAGTPTTPTPTPTPTPTPTTTCVRDSTRDHVAADRAVSLYGRA YARGSRDSLGLATSSYNVVSLOQVEGGWRLVTAC	6
YebF-AmCUT	<i>Amycolatopsis sp.</i> ATCC 39116	meknmkkrqafllglllvsacasvfaannetsksvtfpkcedldaagiaas vkrdyqqnrvarwaddqkivgqadpvawvslqdiqgkddkwsvpltvrgk sadihyqvsdckagmaeyqrrACSDLEIVFARGSGEAPGLGITGTPLVS DVKSALSSASVSSYAVDYAASYDQTSAGPGATDMSNHKSTAAACPDTKF AIGGYSQGASVTDIAIGIRTYLGTGTQTIPTELAPRVVAVIAFGNPLGLYG QTIKTASPTYGPKSLEFCNRGDNVCGGTGTGPGYGHLSYARDGSVDQAAA FIAKQYNAS	26
YebF-Am-Kr	<i>Amycolatopsis sp.</i> ATCC 39116; <i>Kineococcus radiotolerans</i>	meknmkkrqafllglllvsacasvfaannetsksvtfpkcedldaagiaas vkrdyqqnrvarwaddqkivgqadpvawvslqdiqgkddkwsvpltvrgk sadihyqvsdckagmaeyqrrACSDLEIVFARGSGEAPGLGITGTPLVS DVKSALSSASVSSYAVDYAASYDQTSAGPGATDMSNHKSTAAACPDTKF AIGGYSQGASVTDIAIGIRTYLGTGTQTIPTELAPRVVAVIAFGNPLGLYG QTIKTASPTYGPKSLEFCNRGDNVCGGTGTGPGYGHLSYARDGSVDQAAA FIAKQYNASACTPTTPTPTPTPTPTPTTTCVRDSTRDHVAADRAVSLYGRA YARGSRDSLGLATSSYNVVSLOQVEGGWRLVTAC	27
YebF-CfCUT	<i>Cellulomonas flavigena</i>	meknmkkrqafllglllvsacasvfaannetsksvtfpkcedldaagiaas vkrdyqqnrvarwaddqkivgqadpvawvslqdiqgkddkwsvpltvrgk sadihyqvsdckagmaeyqrrAPACPDVELVFARGTGEAAGLGIVGRPL ERALAELPGRTVVATAVDYAASSSQASAGPGSGDMVAKVRSRAACPCT QFVLGGYSQGATVTDIALGIRTGVTAGTALPEDLAARVAADVVGYNPLGL TRRTIAQAAPAFATRTVEYCNAGDPVCEPGGGRFTHITYATNGTVLEGA RFAAARVTAAPPTPTPGPTGTAPVPSAEPTAPAGDTCVTASSLQHVRDG RAYPLWMRTYARGSGDPLGLVLSRTTVVSLQADGTDTRKVAAC	28

YebF-Cf-TP	<i>Cellulomonas flavigena</i> ; <i>Kineococcus radiotolerans</i>	meknmkkrgaflglllvscasvfaannetsksvtfpkcedldaagiaas vkrdyqqnrvarwaddqkivgqadpvawvslqdiqgkddkwsvpltvrgk sadihyqvsdckagmaeyqrrAPACPDVELVFARGTGEEAAGLGIVGRPL ERALAAELPGRTVVATAVDYAASSSQASAGPGSGDMVAKVRSRAACP GTQFVLGGYSQGATVTDLALGIRTGVTAGTALPEDLAARVAAVVYGNPLGL TRRTIAQAAPAFATRIVEYCNAGDPVCEPGGGRFTAHTYATNGTVLEGA RFAAARVTAAGTPTTPTPTPTPTPTPTTCTASSLQHVDRGRAYPLWMRT YARGSGDPLGLVLSRTTVVSLQADGTDTRKVAAC	29
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The constructed and expressed enzymes include the predicted mature wild-type cutinases from *Kineococcus radiotolerans* ("KrCUT"), *Amycolatopsis* sp. ATCC 39116 ("AmCUT", which lacks a linker domain and a polymer binding domain), and *Cellulomonas flavigena* ("CfCUT"), as well as the chimeric variants "YebF-Am-Kr" (having AmCUT fused to the T/P-rich linker domain and the polymer binding domain of KrCUT), and "YebF-Cf-TP" (having the cutinase catalytic domain of CfCUT, the T/P-rich linker domain of KrCUT, and the polymer binding domain of CfCUT). The domain structures of each of the recombinant enzymes listed in **Table 8** are summarized in **Table 9** (following autolytic cleavage of the respective YebF carrier proteins).

Table 9. Summary of domain structure of recombinant enzymes having cutinase activity

Enzyme name	Cutinase catalytic domain from:	Linker domain from:	Polymer binding domain from:
KrCUT	<i>Kineococcus radiotolerans</i>	<i>Kineococcus radiotolerans</i>	<i>Kineococcus radiotolerans</i>
AmCUT	<i>Amycolatopsis</i> sp. ATCC 39116	n/a	n/a
Am-Kr	<i>Amycolatopsis</i> sp. ATCC 39116	<i>Kineococcus radiotolerans</i>	<i>Kineococcus radiotolerans</i>
Cf	<i>Cellulomonas flavigena</i>	<i>Cellulomonas flavigena</i>	<i>Cellulomonas flavigena</i>
Cf-TP	<i>Cellulomonas flavigena</i>	<i>Kineococcus radiotolerans</i>	<i>Cellulomonas flavigena</i>

Constructions

To ensure expression of the recombinant enzymes, synthetic genes encoding fusion proteins consisting of the carrier protein YebF (357 bp) and the corresponding full-length cutinases lacking the native N-terminal signal sequences were constructed.

The optimized sequences for reduction of G+C content were provided by Biomatik USA. The nucleotide sequences were cloned into the *NcoI* and *HindIII* sites of the pTrc2Bd vector, a derivative of the plasmid pTrcHis2B, obtained by deletion of the His Tag. The recombinant plasmids were transformed in the β -galactosidase-positive and lactose inducible *E. coli* MG1655 strain.

The synthetic gene of *Amycolatopsis* sp. ATCC 39116, cutinase (AmCUT) corresponds to 564 bp, after optimization the G+C content was reduced from 71% to 55%.

The recombinant enzyme variant Am-Kr was constructed as a 786 bp polynucleotide consisting of 561 bp AmCUT and 225 bp of the C-terminal portion of KrCUT. Codon optimization led to G+C content reduction from 68% to 56%.

The synthetic gene encoding CfCUT corresponded to an 812 bp nucleotide sequence, in which G+C content was reduced from 78% to 59% after codon optimization.

The polynucleotide encoding the variant CfTP represented a DNA fragment of 789 bp.

Production at small scale

Expression of the recombinant enzymes were performed by growing the *E. coli* MG1655 harboring the recombinant plasmids pTrc3Bd. Colonies of the cells on LB agar plates were selected and used to prepare pre-cultures consisting of 5 mL TB media supplemented with ampicillin (50 µg/mL). The culture was incubated at 37°C, on a rotary shaker at 200 rpm, for 16 h. The overnight culture was used to inoculate 1 L of fresh TB media. Recombinant enzyme production was induced by addition of 10 g/L lactose, at optical density (OD) 0.5, cells were allow to grow for 24 h, collected by centrifugation at 4000 rpm, 20 min (Eppendorf Centrifuge 5810R). The supernatant represents the source of cutinase for activity measurements.

Production of cutinase variants in Bioreactor

Fermentation was performed in a 30-Litre computer-controlled, in-situ sterilizable bioreactor (INFOR HT Techfors), with a working volume of 20 L. The bioreactor was filled with 18 L of TB culture medium and sterilized at 121°C for 20 min. A regulation system was used to control the temperature at 30°C throughout the experiment. A pH of 7.0 was maintained constant by using acidic H₂SO₄ and basic NaOH regulation.

The stirring speed was first set at 400 rpm and then linked to dissolved oxygen (DO) concentration at 25% saturation. The airflow velocity was set to 1 vvm. The bioreactor was inoculated with 5% volume of culture under aseptic conditions, using pre-culture. First pre-culture was performed in 5 mL TB media, supplemented by ampicillin (50 µg/mL), and incubated at 37°C, 16h, using bacteria colonies from LB agar plate. This culture was used to inoculate the second pre-culture, in a 1L flask, TB medium, incubation under the same conditions.

Expression of recombinant enzymes was initiated at OD₆₀₀ of 7, by adding lactose at a concentration of 10 g/L. The cultivation was allowed to continue for 24 h, after induction, fermentation liquid was collected by centrifugation, 8000 rpm for 30 min (Beckman centrifuge, Model Avanti J26 XPI).

The following crude culture supernatants were obtained:

- KrCUT (~8 L); 1.6 U/mL (pNP-dodecanoate);
- AmCUT (~8 L); 1.2 U/mL (pNP-dodecanoate);
- Am-Kr (~16 L); 0.5 U/mL (pNP-dodecanoate);
- CfCUT (~16 L); 0.5 U/mL (pNP-dodecanoate); and
- Cf-TP (~8 L); 0.6 U/mL (pNP-dodecanoate).

Purification

In a first step, all 5 enzymes were concentrated to about 0.5 L using a 10 kD cut-off holo fiber membrane. During this step, the enzyme containing solutions became much darker, probably due to medium ingredients that remained loosely bound to proteins contained in the solution and thus did not pass the 10 kD barrier.

After concentration, each enzyme solution was subjected to dynamic dialysis on a SPECTRAPOR™ 500-016 HP-75 dialysis module, 10 kD, 6500 cm². In this step, the individual protein concentration was kept constant, but salt and medium components were removed gradually. The enzyme-containing solution was dialyzed against 20 mM sodium phosphate, pH 6.0 until the conductivity of the solution was lower than 2 mS/cm².

- 5 Theoretical isoelectric points and the molecular masses of the enzymes were calculated using their putative amino acid sequences. The results and properties of each of the enzyme variants are shown in **Table 10**.

Table 10. Properties of recombinant enzymes expressed

Enzyme	Length (aa)	Calc. MW (kD)	1 microgram = (pmol)	Molar extinction coefficient	1 Absorbance Unit [280 nm] corr. to (mg/mL)	Absorbance [280 nm] of 1 mg/mL	Isoelectric point	Predicted charge at pH 7
YebF-KrCUT	393	39.5	25.31	37920	1.04	0.96	8.81	6.13
KrCUT	261	26.1	38.37	16650	1.57	0.64	8.59	3.07
YebF-AmCUT	309	32.3	30.98	34550	0.93	1.07	6.98	-0.03
AmCUT	187	18.8	53.11	13280	1.42	0.71	5.20	-3.10
YebF-Am-Kr	383	40	25.0	44320	0.90	1.11	8.04	2.04
Am-Kr	261	26.6	37.65	23050	1.15	0.87	6.32	-1.03
YebF-CfCUT	393	40.6	24.60	42320	0.96	1.04	8.69	4.97
CfCUT	271	27.2	36.77	21060	1.29	0.77	8.29	1.90
YebF-Cf-TP	384	39.9	25.06	42330	0.94	1.06	8.94	6.97
Cf-TP	262	26.5	37.80	21060	1.26	0.80	8.84	3.90

- 10 Based on their calculated molecular weights, their isoelectric points, and other properties, the enzymes YebF-KrCUT and KrCUT, YebF-CfCUT and CfCUT, as well as YebF-Cf-TP and Cf-TP, were all predicted to bind to a cation exchanger (Capto™-S at a pH of 6.0 and a conductivity below 2 mS/cm²). The enzyme YebF-AmCUT was predicted to weakly bind to the cation exchanger, but not the enzyme AmCUT. The enzyme Yeb-F-Am-Kr was predicted to bind to the cation exchanger, whereas the enzyme Am-Kr was considered to bind minimally.

- 15 Despite the generally positive binding assessment of the above enzymes (with exception of AmCUT) to a cation exchanger (Capto™-S and SP-Sepharose™ XL were tested) under the selected conditions (pH 6.0, 2mS/cm²), only KrCUT bound completely to the resin. CfCUT did not bind at all and appeared completely in the flow through, whereas active enzyme for AmCUT, Am-Kr, and Cf-TP were found in both the column flow through and in bound fractions.

- 20 Bound fractions of each enzyme (with the exclusion of CfCUT) were concentrated using stirring cell with a cut-off membrane of 10 kD, and subjected to size exclusion chromatography using a Hi-Load™ Superdex™ pg 16/60 column, which also allowed the assessment of the native molecular mass of the enzymes based on their retention time.

For **KrCUT** separated on SP-Sepharose™ XL, two active fractions (A7/A8, and A11-B1; chromatogram on shown) were collected. Both fractions showed similar activity but, due to the higher yield, fraction A11-B1 was selected for further studies and purified by size exclusion chromatography.

For **AmCUT** separated on Capto™-S, only a very small amount of active enzyme bound to the column and was subsequently concentrated and subjected to size exclusion chromatography (chromatogram not shown). In parallel, the flow through containing most of the active Am enzyme was collected, concentrated, dialysed against 20 mM Na-phosphate, pH 7.0, and applied to a DEAE-Sepharose™ FF 16/10. Under the chosen conditions, active enzyme did not bind to the column and appeared in the flow through that was collected, concentrated and subjected to size exclusion chromatography. Even though AmCUT did not bind to DEAE-Sepharose™, most of the contaminating proteins did and thus this step resulted in an almost pure enzyme. Active almost pure AmCUT was thus only found in fraction X1 (flow through). All bound fractions did not contain enzyme activity.

For **Am-Kr** separated on Capto™-S, in contrast to AmCUT, Am-Kr did bind to the cation exchanger in much larger quantity, although active enzyme was also found in the flow through. Fractions B3-B9 were active and were pooled (chromatograph not shown), concentrated and subjected to size exclusion chromatography as final purification step.

For **CfCUT** separated on Capto™-S, CfCUT did not bind to the cation exchanger at all. Thus, a similar purification approach was chosen as for AmCUT cutinase. The active flow-through of CfCUT was collected, concentrated and diafiltrated against 20 mM sodium phosphate pH 7.0, and the enzyme was loaded to a DEAE-Sepharose™ FF column (16/10). The flow through contained the active enzyme and was collected, concentrated and subjected to size exclusion chromatography on Superdex™ 75 pg. Active **CfCUT** was exclusively found in fraction X1 (chromatograph not shown). None of the bound fractions contained active enzyme.

For **Cf-TP** separated on Capto™-S, in contrast to CfCUT, parts of Cf-TP did bind to the cation exchanger, although active enzyme was also found in the flow through. Fractions B3-B7 (chromatograph not shown) were active and were pooled, concentrated and subjected to size exclusion chromatography as final purification step.

In summary, using different techniques for enzyme purification, pure enzyme preparations were obtained and tested for their activity towards a standard esterase substrate, pNP-palmitate. Enzymatic activities of the purified variants are summarized below:

KrCUT	197 ± 3 U/mg
AmCUT	244 ± 18 U/mg
Am-Kr	174 ± 11 U/mg
CfCUT	26 ± 1 U/mg
Cf-TP	30 ± 2 U/mg

The active fraction of **KrCUT** (Superdex™ 75) was shown to have a native molar mass of about 28 kD (deducted from its retention time, using a standard curve), showing a single band in SDS-PAGE at about 27 kD (**Figure 13A**), which corresponds well with the theoretical molar mass for full length Kr (26 kD, after YebF cleavage).

AmCUT and **Am-Kr** behaved unusual in size exclusion chromatography, which made determining their native molar masses impossible, as the retention time of both enzymes did not correspond to their predicted molar mass. Instead, both enzymes were retained on the column, suggesting some interaction of the enzymes with the column matrix. SDS-PAGE of Am-Kr (**Figure 13B**) revealed two distinct species with molar masses of about 40 kD (which is in agreement with the predicted molar mass for YebF-Am-Kr) and about 22 kD which is slightly below the predicted molar mass of full length Am-Kr (YebF removed, 26 kDa).

Example 19:

Circular dichroism spectroscopy and estimation of melting temperature (T_m)

The enzymes purified in **Example 18** were characterized to determine their melting temperatures.

19.1 Methods

CD spectra were recorded using a Jasco™ J-815 spectrometer operating with the Spectra™ Manager software. Temperature was controlled using a Jasco™ PFD-452S peltier unit. Previously purified protein solutions were adjusted to 0.15 mg/mL (if quantities allowed for) and CD spectra were recorded between 190 and 250 nm using a Quartz cuvette (ID = 0.1 cm). Blanks containing buffer only were prepared and used as baseline. Temperature dependent protein unfolding was monitored at 222 nm with thermo profiles ranging from 20 up to 90 °C (2°C min⁻¹). Samples were kept for 5 min at the respective maximum temperature and protein refolding was monitored using the same conditions as above reversing the thermo profiles. Thermodynamic parameters (T_m, ΔH, ΔS, ΔG) for the folding/unfolding process, if applicable, were calculated using the Spectra™ Manager software.

19.2 Results

Variable temperature measurement using CD spectroscopy (monitored at 222 nm) shows the unfolding of **KrCUT** with increased temperature (**Figure 14A**). The melting temperature (T_m) was estimated to be 50.7°C ±0.1. KrCUT also showed refolding to some extent (**Figure 14A**, upper curve, going from high to low temperature) with a midpoint transition temperature of 42.7°C ±0.2 .

The T_m of **Am-Kr** was estimated to be 55.0°C ±0.1 (**Figure 14B**). In contrast to KrCUT, Am-Kr did not show any refolding.

The T_m of **CfCUT** was estimated to be 65.2°C ±0.1 (**Figure 14C**). CfCUT did not show any refolding.

The CD signal of **Cf-TP** was a slightly low, probably due to lower protein concentration. The T_m of Cf-TP was estimated to be 56.6°C ±0.1 (**Figure 14D**). Cf-TP showed some refolding, but to a much lower extend when compared to KrCUT.

The CD signal of **AmCUT** was too weak to allow for the estimation of a T_m for this enzyme.

All enzymes showed the typical spectrum of alpha-helix dominant secondary structure, as expected being hydrolases.

Example 20:

Enzyme substrate profiles

The enzymes purified in Example 18 were characterized to determine their substrate profiles on common esterase substrates.

20.1 Methods: Esterase assays

Esterase activity was determined by monitoring the formation of para-nitrophenol (pNP) at 410 nm from various pNP-esters using a Beckman UV spectrophotometer (model DU 640). Substrates were completely dissolved in 2-propanol (8 mM) and were then added to 50 mM Tris/HCl buffer pH 8.0 to give a final substrate concentration of 0.8 mM. The reaction (1 mL) was started by adding an appropriate amount of enzyme. One enzyme unit (U) was defined as the amount of enzyme that produced 1 µmol of product (pNP) per min; specific activity was expressed as U/mg protein. A molar extinction coefficient of 15 mM⁻¹ cm⁻¹ was used for the calculation.

20.2 Results

Table 11: Substrate profiles of enzymes tested

Substrates	KrCUT	AmCUT	Am-Kr	CfCUT	Cf-TP
pNP-acetate (C2)	2	8	9 ± 1	6	5
pNP-butyrate (C4)	26 ± 2	20 ± 2	75 ± 8	93 ± 8	80 ± 3
pNP-caprylate (C8)	25 ± 3	76 ± 2	100*	11 ± 2	33 ± 1
pNP-decanoate (C10)	25 ± 3	80 ± 2	72 ± 2	40 ± 5	66 ± 8
pNP-myristate (C14)	100*	100*	46 ± 8	47 ± 8	40 ± 4
pNP-palmitate (C16)	98 ± 6	78 ± 2	49 ± 6	100*	100*

* Relative activities in % for the respective cutinase, highest activity towards a specific substrate set to 100%

As can be seen in Table 11, the highest enzymatic activity for KrCUT and AmCUT was achieved with pNP-myristate, whereas the CfCUT and Cf-TP variants showed the highest activity towards pNP-palmitate. In contrast, Am-Kr seemed to prefer the lower chain substrates with highest activity towards pNP-caprylate. pNP-acetate seems to be a relatively poor substrate for all variants. KrCUT, AmCUT and Am-Kr showed preference for the longer carbon chain substrates, whereas the CfCUT and Cf-TP substrate profile seemed to be more inhomogeneous, with preference for both pNP-butyrate and pNP-palmitate.

Example 21:

Determination of enzyme optimal temperatures and thermostability

The enzymes purified in **Example 18** were characterized to determine their optimum temperatures.

5 **21.1 Methods: Temperature optimum**

For estimation of the temperature optimum, buffer containing pNP-palmitate (see esterase assay in **Example 20.1**) was pre-incubated to reach the desired temperature, enzyme was then added, and the formation of pNP was assayed. For this experiment, Tris/HCl buffer was replaced with sodium phosphate buffer (50 mM, pH 8.0) to avoid a pH shift at the different temperatures.

10

21.2 Results: Temperature optimum

Table 12. Temperature dependence of the different enzymes tested

Temperature (°C)	KrCUT	AmCUT	Am-Kr	CfCUT	Cf-TP
20	83 ± 5	92 ± 11	50 ± 2	69 ± 2	54 ± 5
25	84 ± 4	100*	61 ± 2	100*	84 ± 9
30	83 ± 4	67 ± 3	69 ± 2	63 ± 7	95 ± 5
35	84 ± 4	27 ± 4	78 ± 1	45 ± 5	100*
40	89 ± 6	15 ± 2	87 ± 2	38 ± 8	89 ± 6
45	100*	-	100*	1	72 ± 7
50	90 ± 4	-	81 ± 10	-	37 ± 6
55	38 ± 3	-	37 ± 5	-	9 ± 1
60	15 ± 5	-	9 ± 1	-	

* Relative activities in % for the respective cutinase, highest activity at a specific temperature set to 100%

15

As shown in the results above, the multi-domain enzyme KrCUT showed enzymatic activity at a broader range of temperatures than single-domain AmCUT and fellow multi-domain CfCUT (despite CfCUT having an higher estimated melting temperature than KrCUT, see **Example 19.2**). Interestingly, fusing the T/P-rich linker domain and the polymer binding domains from KrCUT to the cutinase catalytic domain of AmCUT, resulted in a chimeric enzyme (Am-Kr) having higher enzymatic activity at broader range of temperatures than AmCUT. Furthermore, replacing only the T/P-rich linker domain of CfCUT with the corresponding domain from KrCUT was sufficient to produce a chimeric enzyme (Cf-TP) having enzymatic activity at broader range of temperatures than CfCUT.

20

21.3 Methods: Thermostability

25

Enzyme thermostability was studied over a temperature range of 50-95°C using the purified enzyme preparations. Aliquots were incubated at the desired temperature for 5, 10, 30, 60, and 120 minutes, chilled on ice, and assayed for remaining activity towards p-NP-palmitate (pNPP) at room temperature.

21.4 Results: Thermostability

Table 13. Thermostability of the cutinase variants is shown in the following table and graphs.

Time (min)	50 °C	55 °C	60 °C	65 °C	70 °C
KrCUT*					
5	61 ± 5	45 ± 4	34 ± 4	19 ± 2	-
10	31 ± 4	26 ± 4	12 ± 2	-	-
30	12 ± 2	4	-	-	-
60	6	-	-	-	-
120	-	-	-	-	-
AmCUT*					
5	46 ± 6	21 ± 4	3	-	-
10	18 ± 3	10 ± 2	-	-	-
30	5	-	-	-	-
60	-	-	-	-	-
120	-	-	-	-	-
Am-Kr*					
5	96 ± 3	82 ± 17	19 ± 2	12 ± 1	-
10	85 ± 8	64 ± 3	15 ± 2	-	-
30	75 ± 15	56 ± 8	5 ± 1	-	-
60	79 ± 12	42 ± 8	-	-	-
120	80 ± 9	30 ± 3	-	-	-
CfCUT*					
5	109 ± 3	72 ± 7	70 ± 6	65 ± 5	40 ± 5
10	118 ± 3	75 ± 10	75 ± 7	64 ± 4	37 ± 6
30	129 ± 9	77 ± 9	56 ± 10	55 ± 1	33 ± 3
60	120 ± 6	65 ± 10	40 ± 4	30 ± 5	26 ± 3
120	125 ± 20	58 ± 2	28 ± 4	25 ± 4	15 ± 1
Cf-TP*					
5	68 ± 8	42 ± 4	35 ± 3	31 ± 3	-
10	43 ± 3	-	-	-	-
30	42 ± 3	31 ± 1	30 ± 3	25 ± 3	-
60	-	-	22 ± 4	15 ± 2	-
120	-	21 ± 3	15 ± 3	9 ± 2	-

* Relative activities in % for the respective cutinase, activity set to 100 % at time 0.

The results above show that single-domain enzyme AmCUT seemed to be very unstable at higher temperatures, whereas all the multi-domain enzymes (particularly the CfCUT) showed higher thermostability. The letter variant was also shown to have the highest melting temperature, however, the optimal temperature does not seem to reflect the enzyme's higher thermostability.

Example 22:

Enzymatic activity in the presence of organic solvents

The enzymes purified in **Example 18** were characterized to determine their stability and activity in presence of diverse organic solvents.

22.1 Methods

Enzyme activity in various organic solvents was studied over a concentration range of 1-30% at room temperature. pNP-palmitate was completely dissolved in 2-propanol (8 mM) and then added to 50 mM Tris/HCl buffer pH 8.0 containing up to 30% organic solvent to give a final substrate concentration of 0.8 mM. The reaction was started by addition of enzyme and the formation of pNP was assayed as described above in **Example 20.1**.

22.2 Results

Table 14. Enzymatic activity in the presence of organic solvents

Solvent addition to assay	Solvent conc. (v/v)	Relative enzymatic activity (%)				
		KrCUT	AmCUT	Am-Kr	CfCUT	Cf-TP
Buffer only		100*	100*	100*	100*	100*
Methanol	10%	91 ± 12	116 ± 4	87 ± 10	115 ± 11	121 ± 14
	20%	8	16 ± 2	60 ± 13	62 ± 3	94 ± 1
2-Propanol	5%	55 ± 11	60 ± 2	70 ± 10	70 ± 3	93 ± 1
	10%	8	9 ± 2	23 ± 2	17 ± 8	92 ± 2
Dimethyl sulfoxide (DMSO)	10%	106 ± 4	142 ± 12	114 ± 2	105 ± 11	150 ± 14
	20%	14 ± 2	53 ± 2	90 ± 8	107 ± 1	146 ± 23
	30%	2	7 ± 2	60 ± 5	57 ± 15	129 ± 18
Dimethylformamide (DMF)	5%	55 ± 11	149 ± 7	120 ± 11	101 ± 17	134 ± 10
Acetone	1%	71 ± 2	170 ± 2	118 ± 1	92 ± 9	103 ± 12
	5%	47 ± 7	126 ± 5	90 ± 14	95 ± 1	112 ± 10
	10%	-	26 ± 7	74 ± 7	52 ± 5	109 ± 6

* Relative activities in % for the respective cutinase, activity with buffer only as negative control set to 100 %

The results above show that the chimeric enzymes Am-Kr and Cf-TP impressively exhibited increased tolerance to higher concentrations of most of the organic solvents tested. More particularly, the chimeric Am-Kr enzymes exhibited higher relative activity in the presence of methanol (20% v/v), 2-propanol (10% v/v), dimethyl sulfoxide (DMSO; 20% or 30% v/v), and acetone (10% v/v), as compared to its corresponding wild-type enzymes (KrCUT and AmCUT). Similarly, the chimeric Cf-TP enzyme exhibited higher relative activity in the presence of all the organic solvents tested, as compared to its corresponding wild-type enzymes (KrCUT and CfCUT).

Example 23:**pH dependence of enzymes**

The enzymes purified in **Example 18** were characterized to determine their stability and activity at different pHs.

23.1 Methods: pH optimum

The influence of different pH values (from 5.5 to 11) on the activities of the purified enzymes was examined in three buffer systems (sodium acetate, sodium phosphate, and Tris-HCl) at room temperature using pNP-palmitate (see esterase assay in **Example 20.1**) as a substrate at a final substrate concentration of 0.8 mM.

23.2 Results**Table 15.** pH dependence of each of the enzymes

pH	Relative activity (%)				
	KrCUT	AmCUT	Am-Kr	CfCUT	Cf-TP
6.5	22 ± 1	35 ± 6	23 ± 2	13 ± 2	12 ± 1
7.0	53 ± 2	56 ± 6	46 ± 3	40 ± 4	22 ± 1
7.5	72 ± 4	56 ± 2	54 ± 3	65 ± 5	36 ± 1
8.0	87 ± 6	38 ± 8	84 ± 2	62 ± 2	65 ± 7
8.5	100*	69 ± 4	88 ± 9	85 ± 6	88 ± 8
9.0	96 ± 4	94 ± 4	91 ± 5	93 ± 9	95 ± 3
9.5	76 ± 7	100*	100*	100*	100*
10	37 ± 5	73 ± 2	47 ± 4	31 ± 1	35 ± 4
10.5	-	60 ± 2	10 ± 1	-	-

* Relative activities in % for the respective cutinase, highest activity at a specific pH set to 100%, sodium phosphate buffer (6.5-7.5), Tris/HCl (8.0-10.5)

Example 24:**Stability of enzymes towards oxidizing agent**

The enzymes purified in **Example 18** were characterized to determine their stability and activity after being exposed to an oxidizing agent (i.e., hydrogen peroxide).

24.1 Methods: Stability in the presence of hydrogen peroxide

Purified cutinases were incubated at room temperature in the presence of 0, 30, 100, 300, 1500, and 3000 ppm of hydrogen peroxide. Residual enzyme activity was assayed (see **Example 20.1**) after 1, 2, 3, and 7 days of incubation.

24.1 Results

Table 16. Enzyme stability following exposure to hydrogen peroxide

H ₂ O ₂ (ppm)	Relative activity (%)				
	Day 0	Day 1	Day 2	Day 3	Day 7
KrCUT*					
30	100 ± 9	90 ± 6	75 ± 3	75 ± 8	76 ± 2
100	104 ± 6	91 ± 6	88 ± 6	76 ± 2	66 ± 2
300	102 ± 4	87 ± 2	74 ± 1	63 ± 2	49 ± 4
1,500	102 ± 10	80 ± 4	67 ± 4	44 ± 2	11 ± 1
3,000	98 ± 9	60 ± 2	33 ± 1	20 ± 4	-
AmCUT*					
30	94 ± 6	-	-	-	-
100	94 ± 6	-	-	-	-
300	85 ± 12	-	-	-	-
1,500	65 ± 6	-	-	-	-
3,000	44 ± 10	-	-	-	-
Am-Kr*					
30	92 ± 3	90 ± 15	82 ± 8	71 ± 4	63 ± 5
100	101 ± 3	97 ± 5	83 ± 2	73 ± 5	69 ± 4
300	100 ± 9	88 ± 5	83 ± 2	72 ± 10	61 ± 4
1,500	91 ± 8	97 ± 9	75 ± 15	73 ± 3	60 ± 6
3,000	86 ± 6	68 ± 9	63 ± 4	67 ± 2	55 ± 3
CfCUT*					
30	101 ± 22	89 ± 8	82 ± 8	76 ± 6	72 ± 7
100	92 ± 5	114 ± 15	118 ± 15	92 ± 11	94 ± 7
300	93 ± 12	106 ± 20	118 ± 12	102 ± 14	106 ± 13
1,500	126 ± 1	106 ± 12	109 ± 2	95 ± 4	54 ± 16
3,000	128 ± 18	115 ± 2	99 ± 23	60 ± 6	35 ± 1
Cf-TP*					
30	97 ± 1	89 ± 4	78 ± 3	86 ± 2	65 ± 7
100	98 ± 6	91 ± 3	89 ± 7	94 ± 7	74 ± 6
300	95 ± 6	88 ± 3	85 ± 12	98 ± 3	77 ± 5
1,500	95 ± 9	89 ± 3	88 ± 6	87 ± 4	68 ± 7
3,000	80 ± 2	74 ± 5	77 ± 2	78 ± 5	57 ± 5

* Relative activities in % for the respective cutinase, activity with buffer only as negative control set to 100 % at day 0.

As shown above, all of the multi-domain enzymes that were tested (i.e., KrCUT, Kr-Am, CfCUT, and Cf-TP) retained some level of activity after 1 or more days exposure to hydrogen peroxide, whereas the single-domain enzyme AmCUT did not. Furthermore, chimeric enzymes (Kr-Am and Cf-TP) exhibited increased resistance to

hydrogen peroxide, as compared to their corresponding wild-type multi-domain enzymes (KrCUT and CfCUT). For example, after exposure to 3000 ppm hydrogen peroxide for 7 days, no activity was detected for the wild-type KrCUT enzyme. In contrast, the chimeric enzyme Am-Kr (containing the cutinase catalytic domain of AmCUT on the backbone of KrCUT), retained 55% of its activity under the same conditions. Moreover, the relative activity of CfCUT was reduced from 128% at day 0 to 35% at day 7 of exposure to 3000 ppm hydrogen peroxide. In contrast, the relative activity of the chimeric enzyme Cf-TP (containing the T/P-rich linker domain of KrCUT on the backbone of CfCUT) went from 80% to 57% under the same conditions.

Example 25:

Summary of biochemical properties of the enzymes

A summary of some of the results shown in **Examples 19-24** is provided below.

Table 17. Summary of biochemical properties of the tested enzymes

Enzyme	Mol. Mass (native) ¹	Mol. Mass (SDS)	Temp. opt. (°C)	pH opt.	Spec. Act. (U/mg) ²	Tm (°C) ³
KrCUT	28,000	27,000	45	8.5	197 ± 3	51 ± 1
AmCUT	n.a. ⁴	20,000	25	9.5	244 ± 18	n.d. ⁵
Am-Kr	n.a. ⁵	22,000	45	9.5	174 ± 11	55 ± 1
CfCUT ⁶	53,000	27,000	25	9.5	26 ± 1	65 ± 1
Cf-TP ⁷	42,000	25,000	35	9.5	30 ± 2	57 ± 1

- 1) Estimated by retention time of the active fraction on Superdex™ 75 pg using a standard curve
- 2) Towards pNP-palmitate (standard assay)
- 3) Melting temperature of the enzyme, estimated using CD-spectroscopy
- 4) Not applicable, Am and Am-Kr showed some interaction with the resin and thus a delayed retention time
- 5) Not determined, Am concentration was too low to give a good signal in CD spectroscopy
- 6) Cf shows a higher molecular mass native than in SDS-PAGE, which may be due to dimer formation
- 7) Cf-TP showed more than one band in SDS-PAGE: 25,000 and 28,000 Da.

Example 26:

Assessment of Ochratoxin A degradation ability by the enzymes

The enzymes purified in **Example 18** were tested for their ability to degrade the mycotoxin Ochratoxin A. In general, the mycotoxin was incubated with different concentrations of the enzyme, and the remaining mycotoxin concentration, as well the respective degradation products, were monitored by LC-MS over time (where possible/applicable).

26.1 Methods: Mycotoxin testing

Reactions were performed in 1 mL volumes in glass HPLC vials comprising the mycotoxin to be analyzed and the enzyme to be tested. For Ochratoxin A (*Petromyces albertensis*, O1877 Sigma), 50 µL of a 100 ppm stock solution in methanol (final concentration of 5 ppm) was added to 910 µL of distilled water and 40 µL of the enzyme to be tested. In control reactions, the volume of enzyme was substituted with distilled water. The reactions were

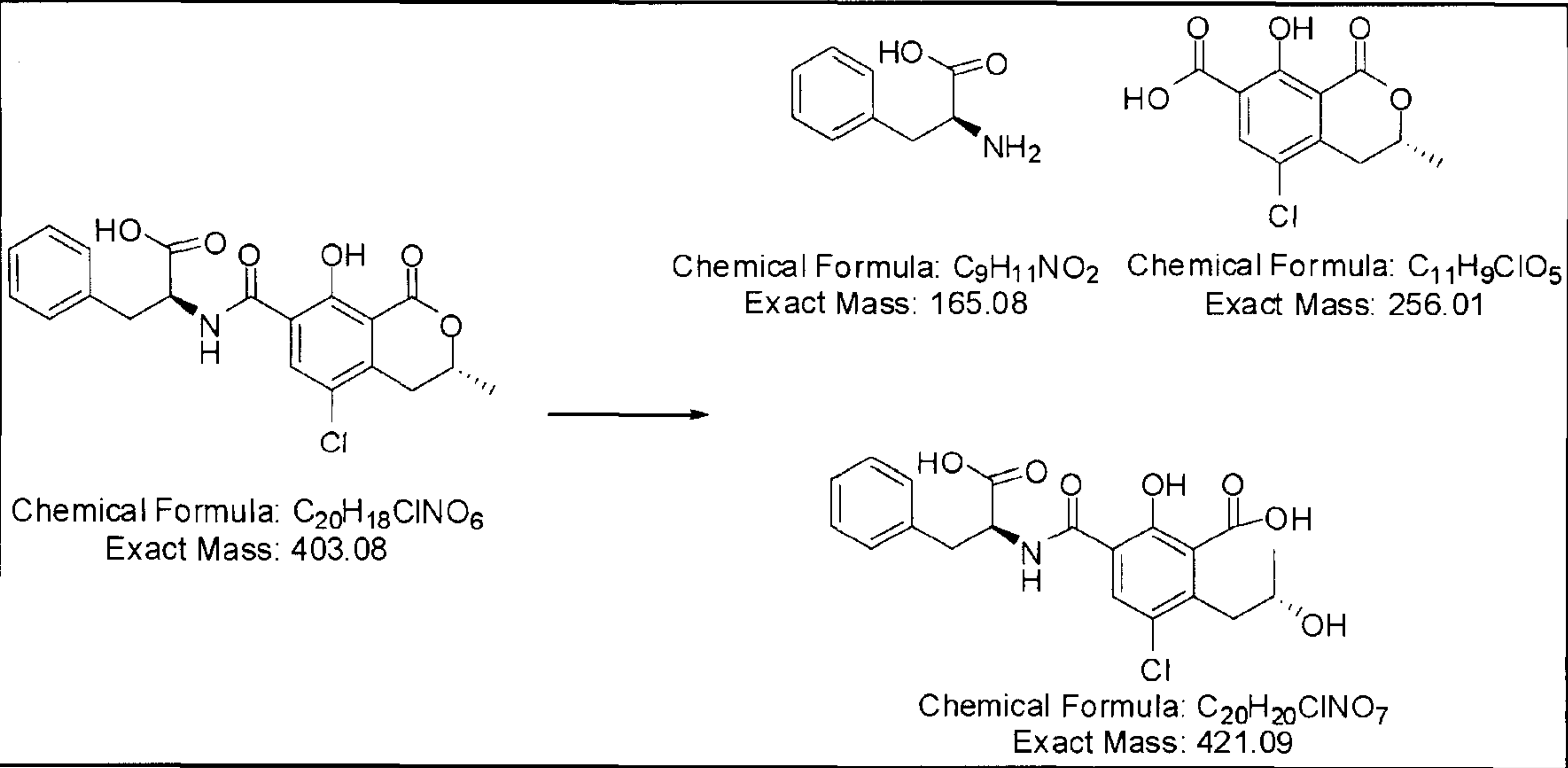
incubated in an Eppendorf thermomixer at 37°C with shaking at 750 rpm. 100 µL samples were withdrawn at 1, 4 and 24 h for chromatographic analysis.

Mycotoxin analysis was carried out using a Shimadzu LCMS-2020 equipped with a UV/vis detector. Chromatographic separation of 10 µL injected sample was carried out at 30°C on a Kinetex 2.6 µm XB-C18 column 100 Å (50 x 2.1 mm) using a binary gradient. For Ochratoxin A, eluent A consisted of water and eluent B consisted of acetonitrile and the UV detection wavelength was 360 nm. Gradient elution at a flow rate of 0.4 mL/min was performed as follows: 0-2 min, 10% B; 2.01-10.0 min, 10 to 80% B; 10.01-12.0 min, 80% B; 12.01-15 min, 10% B. The electrospray interface (ESI) was operated simultaneously in positive and negative mode, scanning from m/z 50 – 1,000.

26.2 Results

The degradation of Ochratoxin A was monitored by HPLC-UV at 360 nm and LCMS. Possible degradation products that would arise from either hydrolysis of the amide bond or hydrolysis of the lactone moiety are shown below.

Chemical structures of Ochratoxin A and hypothetical degradation products



The five enzymes tested were screened for their ability to degrade 5 ppm (5,000 ppb) Ochratoxin A at 37°C. The results are summarized in Table 18.

Table 18. Results of Enzyme testing for Ochratoxin A degradation

Enzyme	Activity (U) ¹	Residual ochratoxin A (%) ²			Ochratoxin A Degradation ³ per U and h (µmol)
		1 h	4 h	24 h	

KrCUT	3.272	63	17	7	0.02
Am-Kr	0.072	6	0	0	3
AmCUT	0.048	59	20	6	2
CfCUT	0.056	11	2	0	3
Cf-TP	0.002	5	0	0	98

- 1) Activity used in assay towards p-nitrophenyl palmitate (standard assay)
2) Relative to Ochratoxin A incubated without enzyme
3) Describes the potency of 1 U of enzyme (pNP standard test) to degrade a specific amount of ochratoxin per hour of incubation at 37°C.

5

Degradation of Ochratoxin A was observed with all enzymes, even after 1 h incubation. The chimeric enzymes AM-Kr and Cf-TP degraded 94-95% of the Ochratoxin A after 1 hours, and 100% after 4 hours. Cf-TP showed the highest “potency” in terms of the amount of Ochratoxin A that 1 U the enzyme (pNP standard test) can degrade per hour of incubation at 37°C.

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Example 27:

Alignment and phylogenic analyses of multi-domain cutinase enzymes

A database search for other multi-domain enzymes using KrCUT (SEQ ID NO: 6) as query sequence revealed five hits, as shown in **Table 19**.

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Table 19. Native cutinase orthologs having multi-domain organization

Enzyme name	Organism	Query coverage	Identity	Accession	SEQ ID NO:
ssKrCUT	<i>Kineococcus radiotolerans</i> SRS30216 = ATCC BAA-149	100%	100%	ABS05574.1	1
ssCbCUT2	<i>Cellulomonas bogoriensis</i> 69B4 = DSM 16987	95%	60%	KGM14008.1	25
ssCcCUT	<i>Cellulomonas cellasea</i> DSM 20118	98%	56%	KGM03336.1	15
ssCbCUT1	<i>Cellulomonas bogoriensis</i> 69B4 = DSM 16987	99%	55%	KGM14009.1	14
ssCfCUT	<i>Cellulomonas flavigena</i> DSM 20109	99%	56%	ADG75999.1	13

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A multiple sequence alignment was performed for the enzymes listed in **Table 19**, after omitting their predicted N-terminal signal sequences, as shown in **Table 20**. The results of the alignment are shown in **Figure 15A**, and a phylogenic tree is shown in **Figure 15B**.

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Table 20. KrCUT orthologs without their predicted N-terminal signal sequences

Enzyme name	Amino acid sequence (cutinase catalytic domains are in bold ; linkers are highlighted in black ; polymer binding domains are <u>underlined</u>)	SEQ ID NO:
KrCUT	ATCSDVDVVFARGTGETPGLGVVGGPFVRSLTGELSDRTVTSHAVDYAASSSQASAGPG ATAMSAHVREVAACPSTRFVLGGYSQGATVTDIALGIRTGTTTGTVPVPAELAGRVA VVFGNPLGLSGRTIATASSTYGPKSKDYCNSSDSVCGSAPKTGTGGHLSYASNGSTTDG ARFAAGLVRAAG TPTTPTPTPTPTPVPTT CVRDSTRDHVAADRAVSLYGRAYARGSRDS <u>LGATSSYNVVSLLQQVEGGWRLVTAC</u>	6
CbcCUT2	AGSCPDVQVVFARGTGERAGLGIIIGRPFARALADELPGMTVTSHAVDYAAAAAASQRSAGP GATAMTDHVTAMAARCPGTQFVLGGYSQGATVTSIALGIRAGTTTGRAIPDELSDRVAA VVVFGNPLGMRGQTIASASRTYADRAKDYCNSGDSICGRQPSTGRGTHGTGYATNGSTTD GARFAAGLVTA NPPEAAPPTAPPTTPPTTPAPP SDQVTCVTAQVSEHVEARRAIRGLTR <u>AYARGTLEDIGRLRSTEEVSLRRSGTFSWTPTASC</u>	46
CcCUT	APACADVEVLFAARGTGEAPGLGVLGTPFVRSVTSALSDRTVTSYAVNYAAESSQRSAGP GATDLTNHLTATAAACPGTRFVLGGYSQGATVVDLALGIRTGTTTGTAIPTAALEPRVAA IVVFGNPLGLISGRTIATASPTYAARARDFCATGDPVCGGGSSFAAHLAYRTNGDVTAGA DFAAGLARAT TVPVPTPTVP GTPTPSPTAPGTPTPVPTTSPTPAPSPTAPGAACVRAST <u>RAHVEAGRAERRHGIAYATGSGDRIGWVSSFVRVSVQQTADGWERVLS</u>	47
CbcCUT1	TGDCPDVHVVFARGTGEPRGLGIVGRPFVSDLGDALPTMTVTSYAVNYSANASQTSAGP GAGDMTSHVTSMAARCPGTQFVLGGYSQGATVTSIAVGARSTSIRSRVLPANLEPRVAA VVVFGNPLGLTRRTIASEAPAYAAKSRDYCNRSDTVCGGRGDARGGHAYVSNGSVADG AAFAA SLVGVAPGDP ASCVTARAVDHVEADRAFRSVLRAYARGSRDPLGRLTSSDLVSL <u>QRTGQDSWSVVPAC</u>	48
CfCUT	APACPDVELVFARGTGEAAGLGIVGRPLERALLAELPGRTVVATAVDYAASSSQASAGP GSGDMVAKVRSRAAACPGTQFVLGGYSQGATVTDLALGIRTGVTAGTALPEDLAARVAA VVVYGNPLGLTRRTIAQAAPAFATRTVEYCNAGDPVCEPGGRFTAHTYATNGTVLEG ARFAAARVTA PAPTPTPGPTGTPAPVPSAEPTAPGDT CVTASSLQHVRDGRAYPLWMR <u>TYARGSGDPLGLVSSRTTVVSLQADGTDTRKVAAC</u>	28

The threonine/proline contents of the T/P-rich linker domains of four of the enzymes listed above are shown in Table 21.

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Table 21. Threonine/proline content of selected multi-domain cutinases

Enzyme name	Amino acid sequence of T/P-rich linker	SEQ ID NO:	# of T or P residues	Linker length	T/P content
KrCUT	TPTTPTPTPTPTPVPTT	3	16	17	94.1%
CbcCUT2	NPPEAAPPTAPPTTPPTTPAPP	37	16	22	72.7%
CcCUT	GLARATTVPVPTPTVPGTPTPSPTAPGT PTPVPTTSPTPAPSPTAPGAA	40	17	28	60.7%
CfCUT	PAPTPTPGPTGTPAPVPSAEPTAPGDT	31	29	49	59%

The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

The scope of the claims should not be limited by the preferred embodiments set forth in the examples, but should be given the broadest interpretation consistent with the description as a whole.

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CLAIMS:

1. An isolated recombinant protein having cutinase activity, said protein comprising:
 - (a) a cutinase catalytic domain;
 - 5 (b) a proline/threonine-rich linker domain positioned C-terminal of domain (a); and
 - (c) a polymer binding domain positioned C-terminal of domain (b).

2. The isolated recombinant protein of item 1, wherein said protein, as compared to a corresponding native protein comprising domain (a):
 - 10 (i) has improved stability to a bleaching agent which is:
 - (1) an oxidizing agent which is at least one of: hydrogen peroxide, a peroxide other than hydrogen peroxide, a non-peroxide oxidizing agent, ozone, sodium percarbonate, sodium perborate, or any combination thereof; and/or
 - (2) a reducing agent which is at least one of: sodium dithionite, sodium dithionite, sulfur dioxide, a sulfite, a bisulfite, a sodium borohydride, or any combination thereof;
 - 15 (ii) has improved stability to an organic solvent which is at least one of: methanol, 2-propanol, or another alcohol, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), acetone, or another organic solvent, or any combination thereof;
 - (iii) has a broader temperature activity range; or
 - 20 (iv) any combination of (i) to (iii).

3. The isolated recombinant protein of claim 1 or 2 which is a chimeric recombinant protein, wherein at least one of domains (a), (b) and (c) is from a cutinase belonging to a different species.

- 25 4. The isolated recombinant protein of any one of claims 1 to 3, wherein domain (a), (b), and/or (c) is from a multi-domain cutinase belonging to *Kineococcus radiotolerans*, *Cellulomonas flavigena*, *Cellulomonas bogoriensis*, or *Cellulomonas cellasea*.

5. The isolated recombinant protein of any one of claims 1 to 4, wherein:
 - 30 (i) domain (a) is:
 - (1) a cutinase catalytic domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to amino acid sequence of SEQ ID NOs: 2, 30, 33, 36, 39, or 42-45, or to the cutinase catalytic domain of SEQ ID NO: 1, 13-20, or 25;

- (2) a cutinase catalytic domain comprising or consisting of the amino acid sequence of SEQ ID NOs: 2, 30, 33, 36, 39, or 42-45, or the cutinase catalytic domain of SEQ ID NO: 1, 13-20, or 25; or
- (3) both (1) and (2);
- 5 (ii) domain (b) is:
- (1) a threonine/proline-rich linker domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to SEQ ID NO: 3, 31, 34, 37, or 40, or to the threonine/proline-rich linker domain of SEQ ID NO: 1 or 13-15;
- 10 (2) a threonine/proline-rich linker domain comprising or consisting of the amino acid sequence of SEQ ID NO: 3, 31, 34, 37, or 40, or to the threonine/proline-rich linker domain of SEQ ID NO: 1 or 13-15;
- (3) a threonine/proline-rich linker domain having at threonine/proline content of at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% over a stretch of 15 to 55 consecutive amino acids; or
- 15 (4) any combination of (1) to (3);
- (iii) domain (c) is:
- (1) a polymer binding domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to SEQ ID NO: 4, 32, 35, 38, or 41;
- 20 (2) a polymer binding domain comprising or consisting of the amino acid sequence of SEQ ID NO: 4, 32, 35, 38, or 41, or to the polymer binding domain of SEQ ID NO: 1 or 13-15; or
- (3) both (1) and (2); or
- (iv) any combination of (i) to (iii).
- 25 6. The isolated recombinant protein of any one of claims 1 to 5, wherein said recombinant protein:
- (i) is produced fused to a heterologous carrier protein or heterologous tag that enables secretion of said recombinant protein, and/or facilitates purification or detection of said recombinant protein;
- (ii) is truncated from a native N terminal signal sequence found on a corresponding native cutinase; or
- 30 (iii) both (i) and (ii).
7. The isolated recombinant protein of claim 6, wherein said carrier protein is YebF as defined by SEQ ID NO:
- 8.

8. A polynucleotide molecule comprising a nucleic acid sequence encoding the recombinant protein as defined in any one of claims 1 to 7, operably linked to a heterologous promoter.

9. The polynucleotide of claim 8, wherein the recombinant protein encoded by said polynucleotide:

- (i) lacks an N terminal signal sequence of a native cutinase;
- (ii) is fused to a heterologous carrier protein; or
- (iii) both (i) and (ii).

10. A vector comprising the polynucleotide molecule as defined in claim 8 or 9.

11. A cell comprising the isolated recombinant protein as defined in any one of claims 1 to 7, the polynucleotide molecule as defined in claim 8 or 9, or the vector as defined in claim 9.

12. The cell of claim 11, which is a bacterial cell, a yeast cell, a non-yeast fungal cell, an insect cell, a plant cell, an animal cell, or a mammalian cell.

13. The cell of claim 12, wherein said cell is from *E. coli*, *Bacillus subtilis*, or *Streptomyces* spp.

14. A composition comprising:

- (i) the isolated recombinant protein as defined in any one of claims 1 to 7; and
- (ii) a bleaching agent and/or an organic solvent,

wherein said recombinant protein retains said cutinase activity when present in said composition.

15. The composition of claim 14, wherein:

(i) said bleaching agent is:

- (1) an oxidizing agent which is or comprises: hydrogen peroxide, a peroxide other than hydrogen peroxide, a non-peroxide oxidizing agent, ozone, sodium percarbonate, sodium perborate, or any combination thereof; and/or
- (2) a reducing agent which is or comprises: sodium dithionite, sodium dithionite, sulfur dioxide, a sulfite, a bisulfite, a sodium borohydride, or any combination thereof;

- (ii) said organic solvent which is or comprises: methanol, 2-propanol, or another alcohol, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), or acetone; or
- (iii) both (i) and (ii).

16. The composition of claim 14 or 15, wherein:

(i) said bleaching agent is present at a concentration of:

(1) at least 100 ppm, 150 ppm, 200 ppm, 250 ppm, 300 ppm, 350 ppm, 400 ppm, 450 ppm, 500 ppm, 550 ppm, 600 ppm, 650 ppm, 700 ppm, 750 ppm, 800 ppm, 850 ppm, 900 ppm, 950 ppm, 1000 ppm, 1500 ppm, 2000 ppm, 2500 ppm, 3000 ppm, 3500 ppm, 4000 ppm, 4500 ppm, 5000 ppm, 5500 ppm, 6000 ppm, 6500 ppm, or 7000 ppm; or

(2) between 100 ppm, 150 ppm, 200 ppm, 250 ppm, 300 ppm, 350 ppm, 400 ppm, 450 ppm, 500 ppm, 550 ppm, 600 ppm, 650 ppm, 700 ppm, 750 ppm, 800 ppm, 850 ppm, 900 ppm, 950 ppm, or 1000 ppm, and 1500 ppm, 2000 ppm, 2500 ppm, 3000 ppm, 3500 ppm, 4000 ppm, 4500 ppm, 5000 ppm, 5500 ppm, 6000 ppm, 6500 ppm, or 7000 ppm;

(ii) said organic solvent is present as a concentration of:

(1) at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% v/v; or

(2) between 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, or 25%, and 30%, 35%, 40%, 45%, or 50% v/v; or

(iii) both (i) and (ii).

17. The composition of any one of claims 14 to 16, further comprising:

(i) an additional recombinant enzyme which is: a lipase, an esterase, a pectate lyase, a pectinase, a cutinase, a cellulase, a hemicellulose, an amylase, or any combination thereof, or another recombinant enzyme;

(ii) a detergent;

(iii) a surfactant;

(iv) a buffer;

(v) a chelator; or

(vi) any combination of (i) to (v).

18. The composition of any one of claims 14 to 17, which is:

(i) a cleaning product, a degreaser, a disinfectant, a bleaching product, or any combination thereof; or

(ii) feed or a feed additive.

19. Use of the isolated recombinant protein as defined in any one of claims 1 to 7, or the composition as defined in any one of claims 14 to 17, for:

(i) cleaning, degreasing, disinfecting, bleaching product, biofilm control, or any combination thereof;

- (ii) for inactivating mycotoxin;
- (iii) bioscouring or other treatment of fabrics or textiles;
- (iv) catalyzing trans-esterification reactions;
- (v) vegetable oil extraction; or
- (vi) enhanced oil recovery.

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20. The use of claim 18 for inactivating ochratoxin.

21. A method for producing the recombinant protein as defined in any one of claims 1 to 7, said method comprising:

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- (i) culturing a suitable host cell expressing a polynucleotide encoding said recombinant protein, wherein said polynucleotide encodes a truncated recombinant protein lacking an N terminal signal sequence; and
- (ii) isolating said recombinant protein.

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22. The method of claim 21, wherein said polynucleotide is operably linked to a heterologous promoter.

23. The method of claim 21 or 22, wherein said polynucleotide encodes said recombinant protein fused to a heterologous carrier protein.

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Figure 1

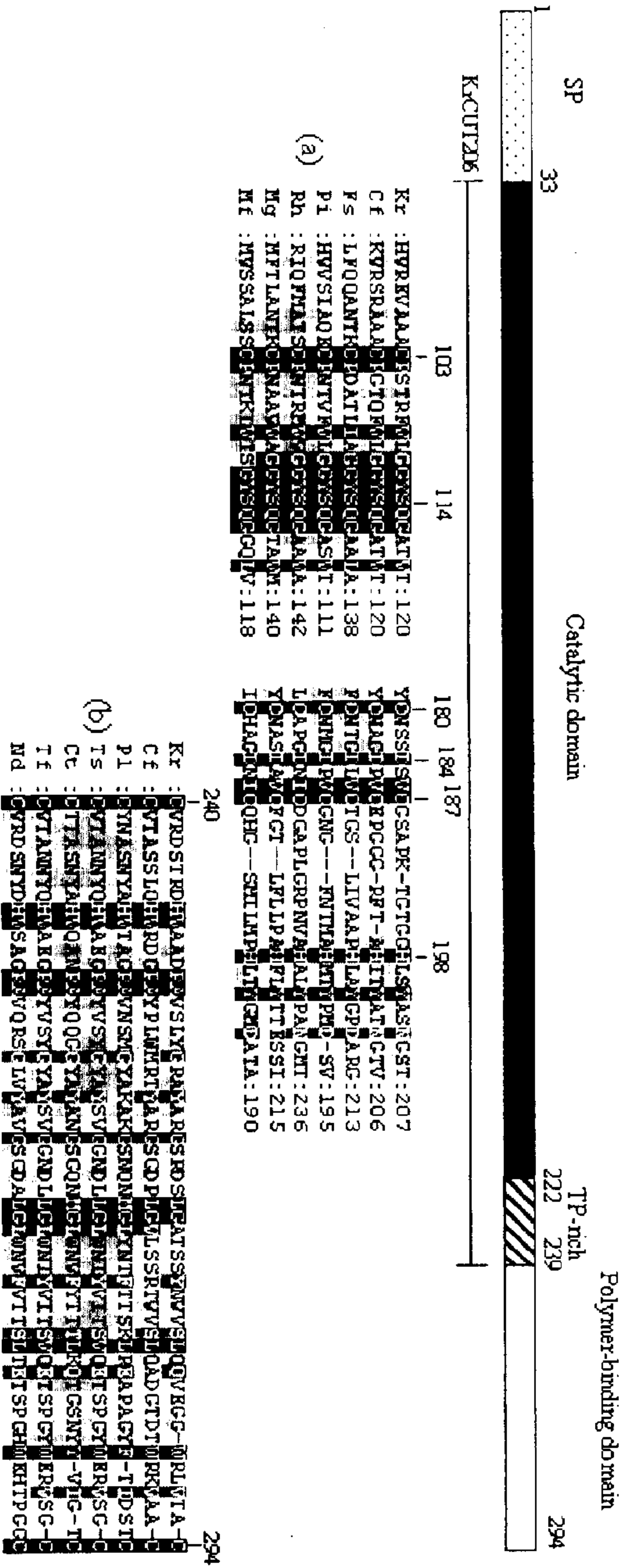


Figure 2

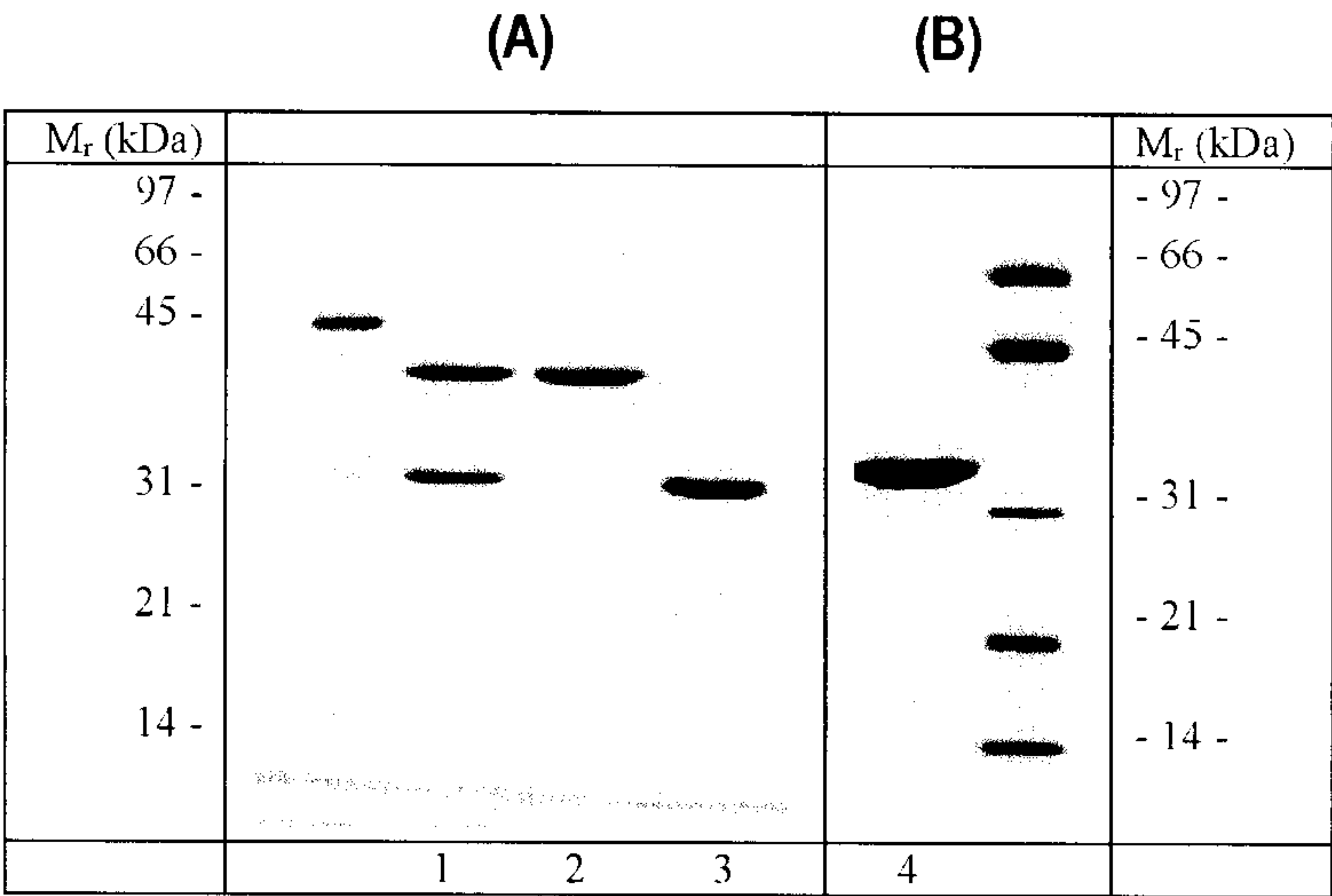


Figure 3

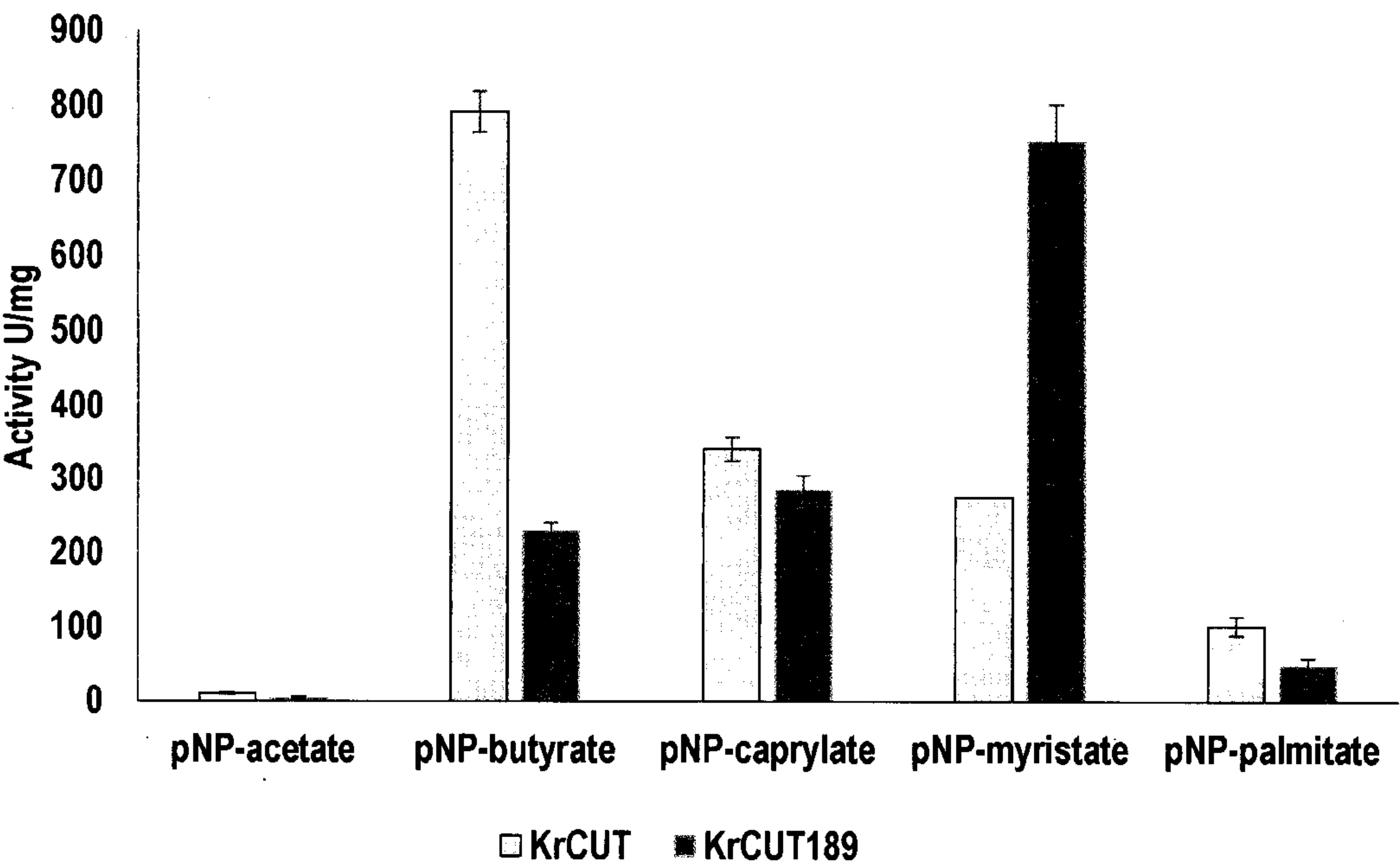


Figure 4

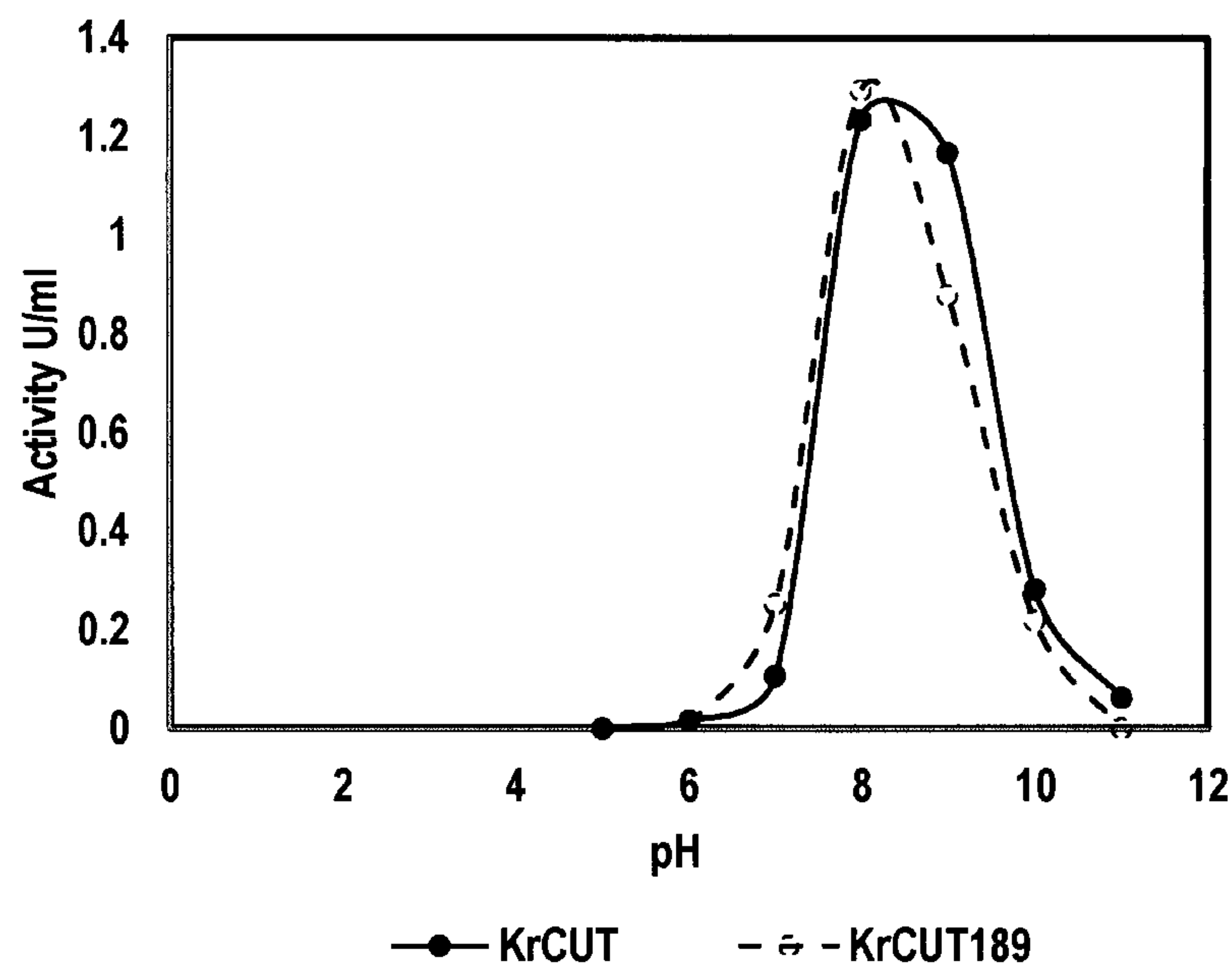


Figure 5

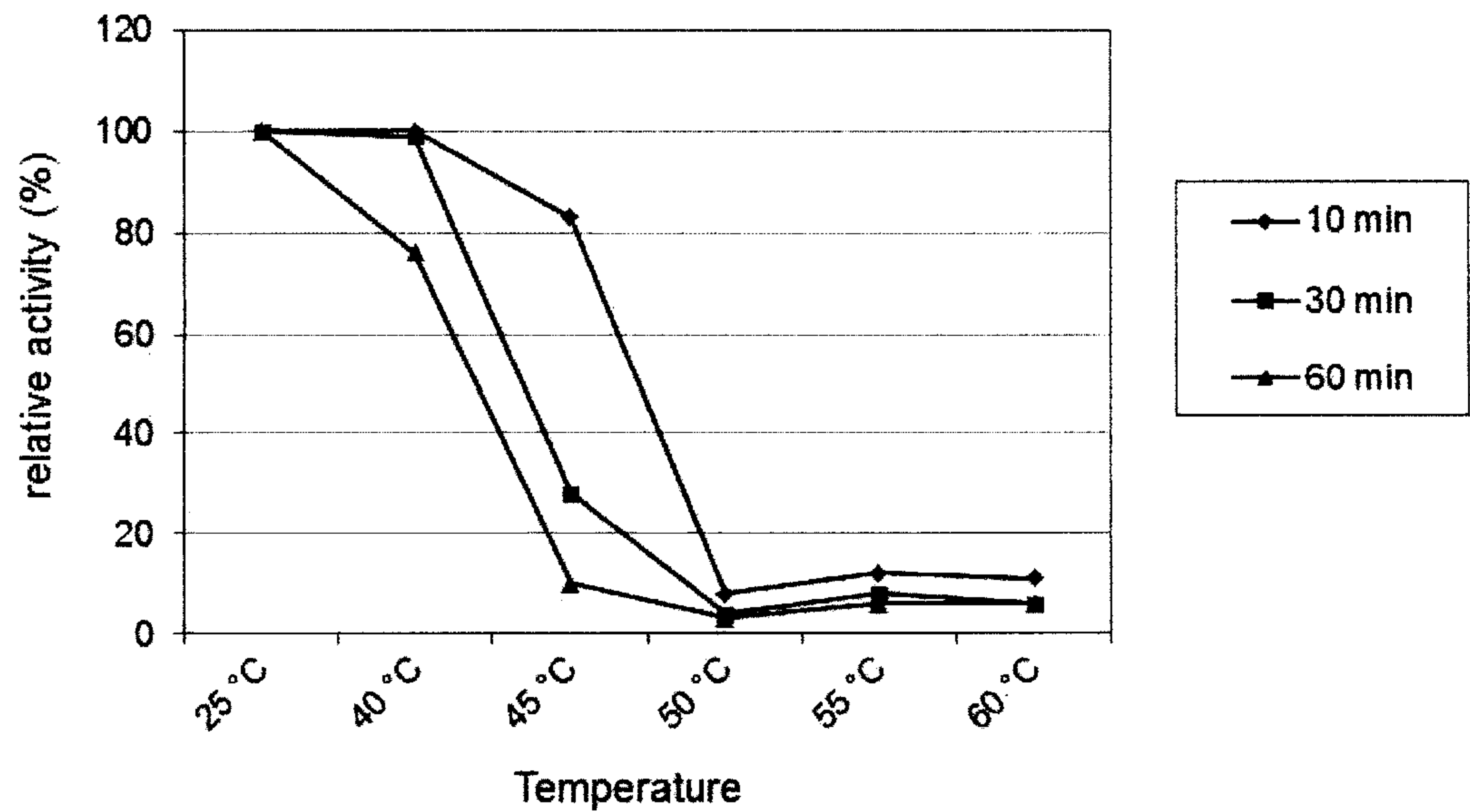


Figure 6

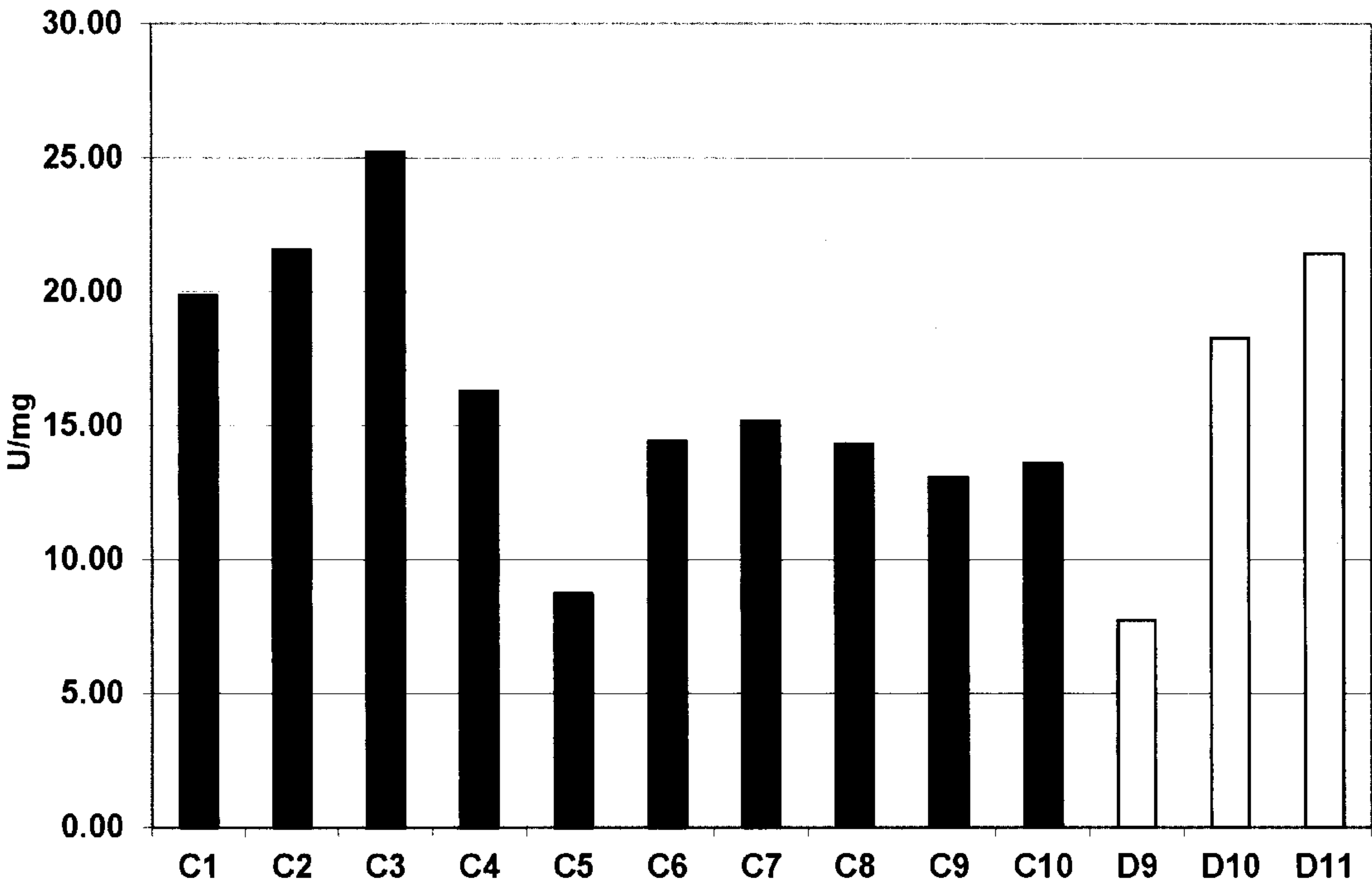


Figure 7

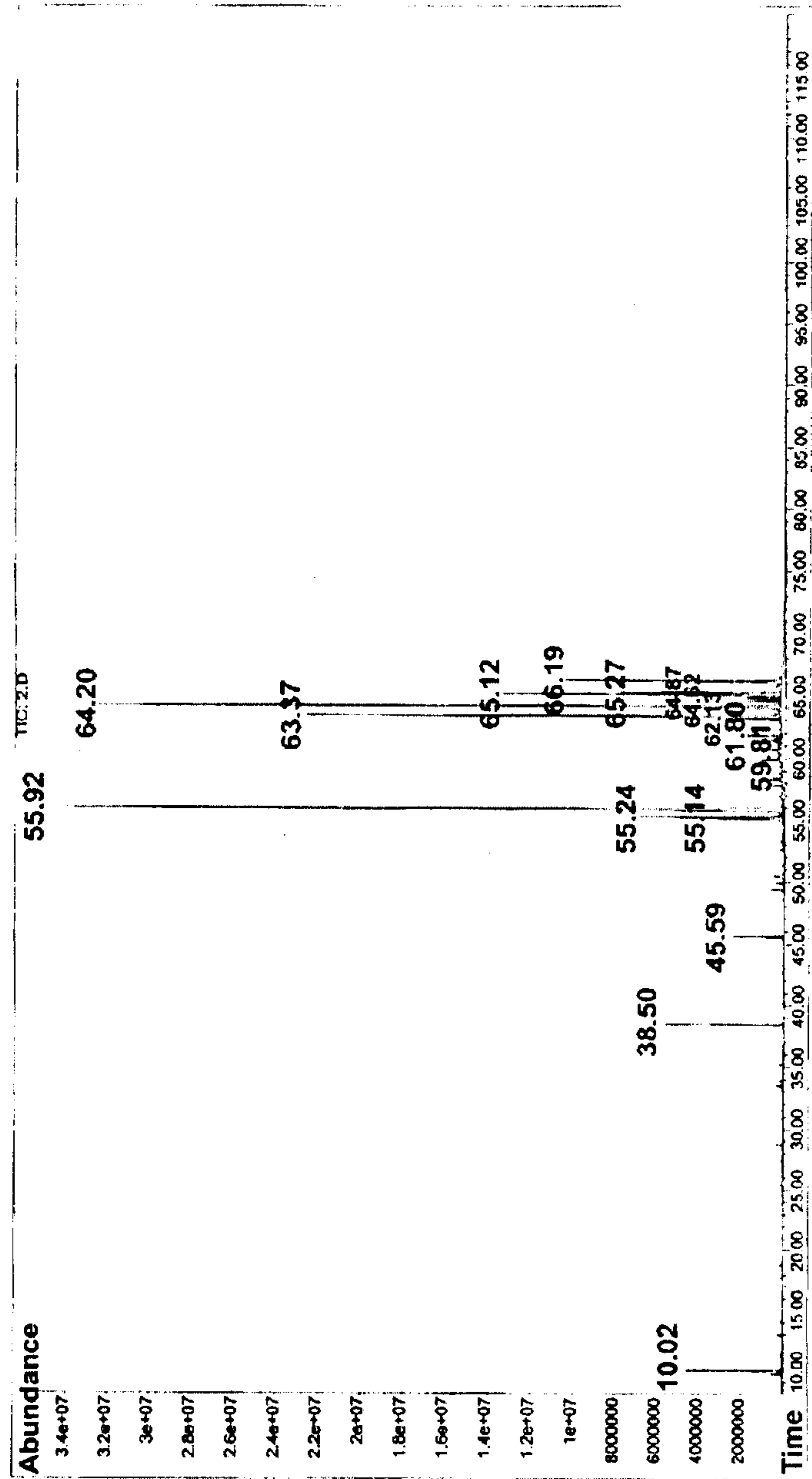


Figure 8

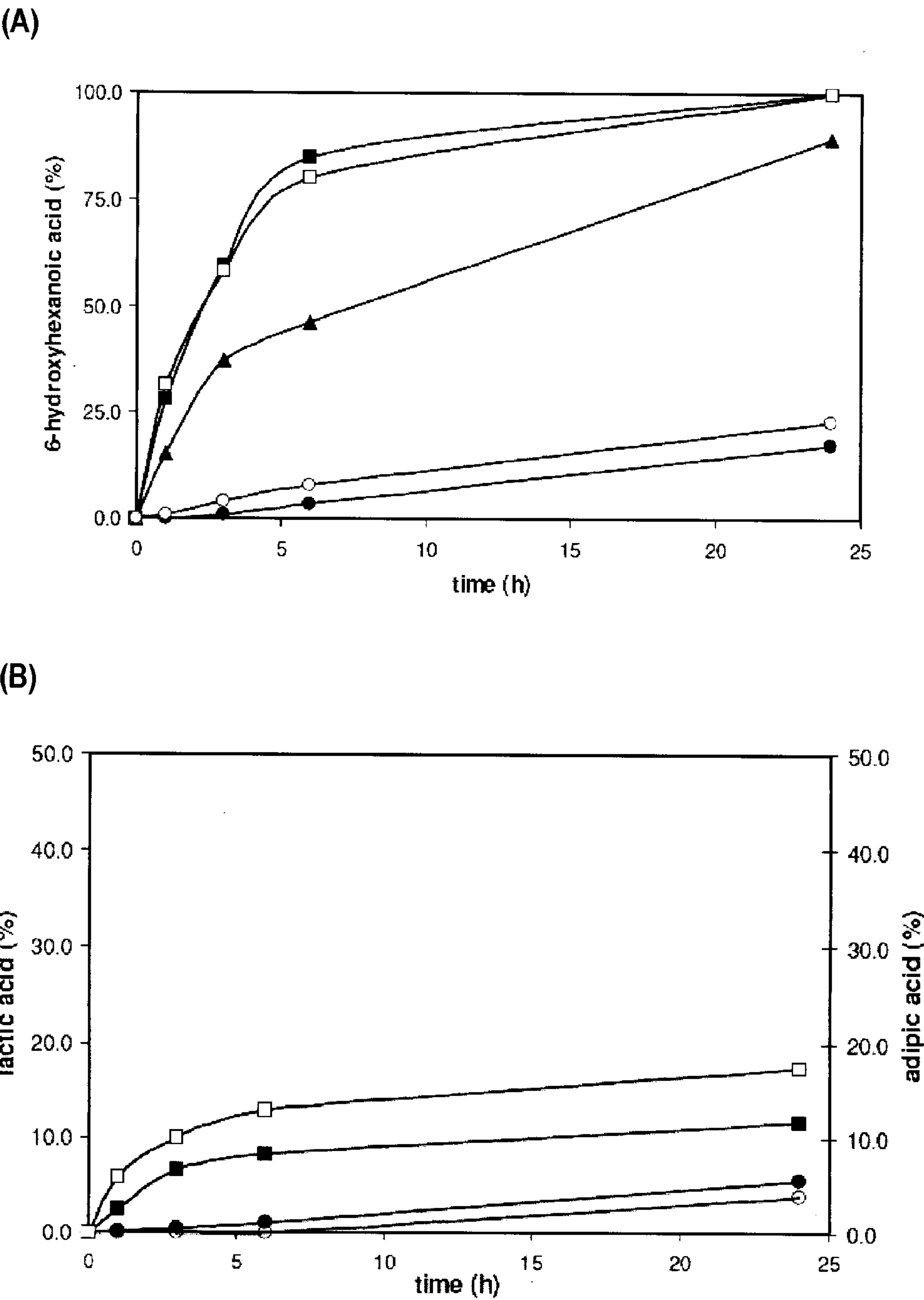


Figure 9

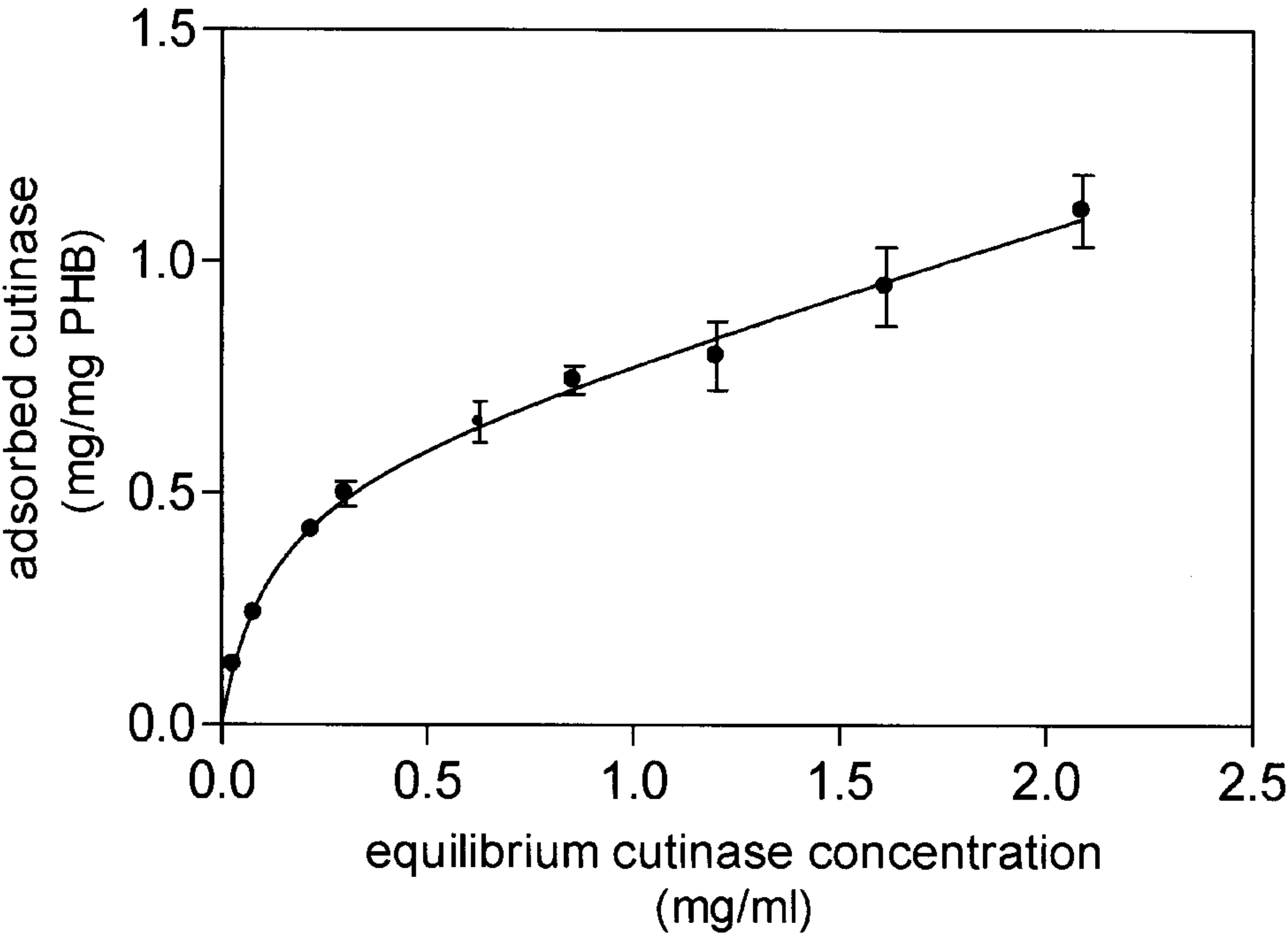


Figure 10

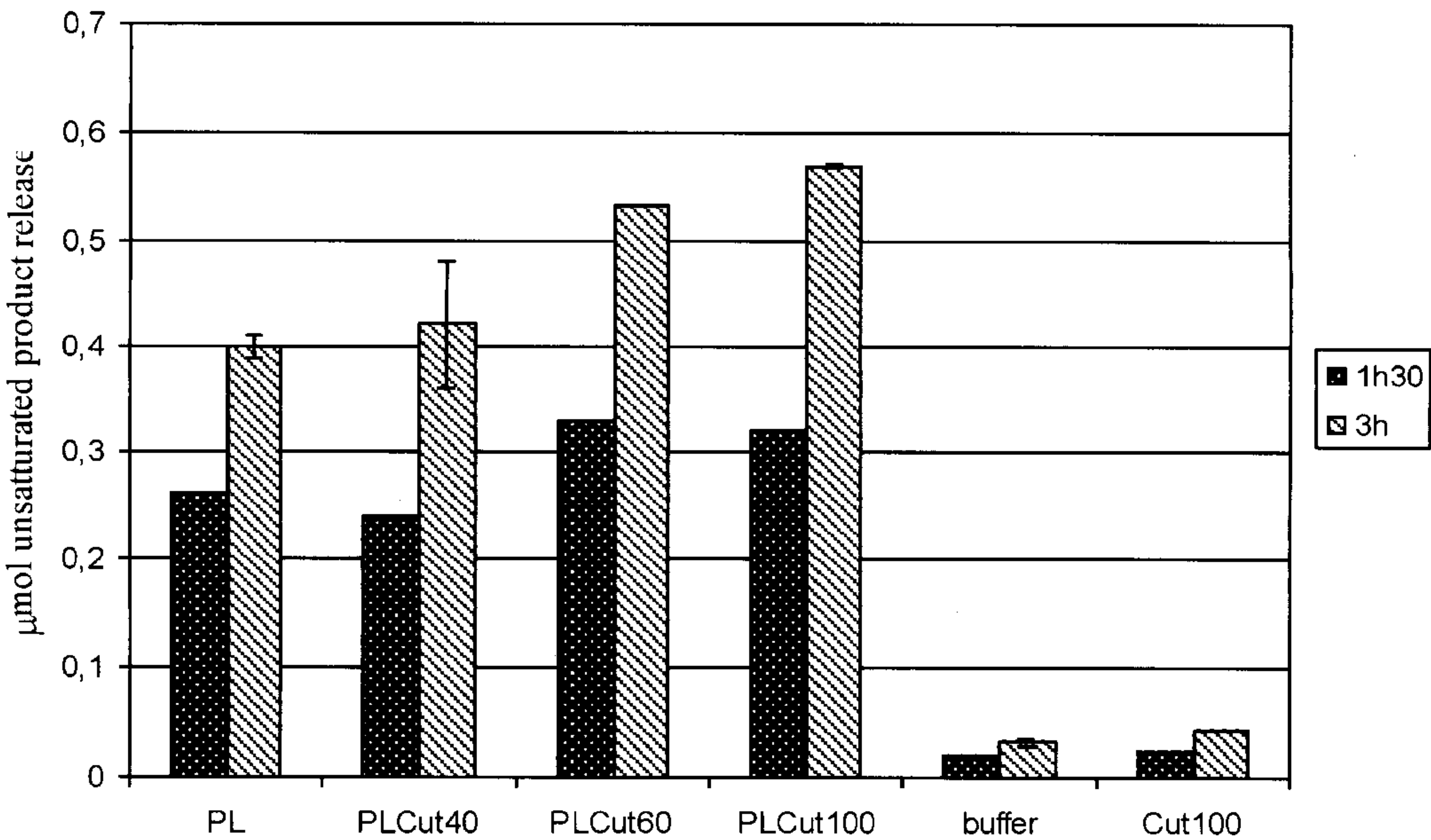


Figure 11



A



B



C



D

Figure 12

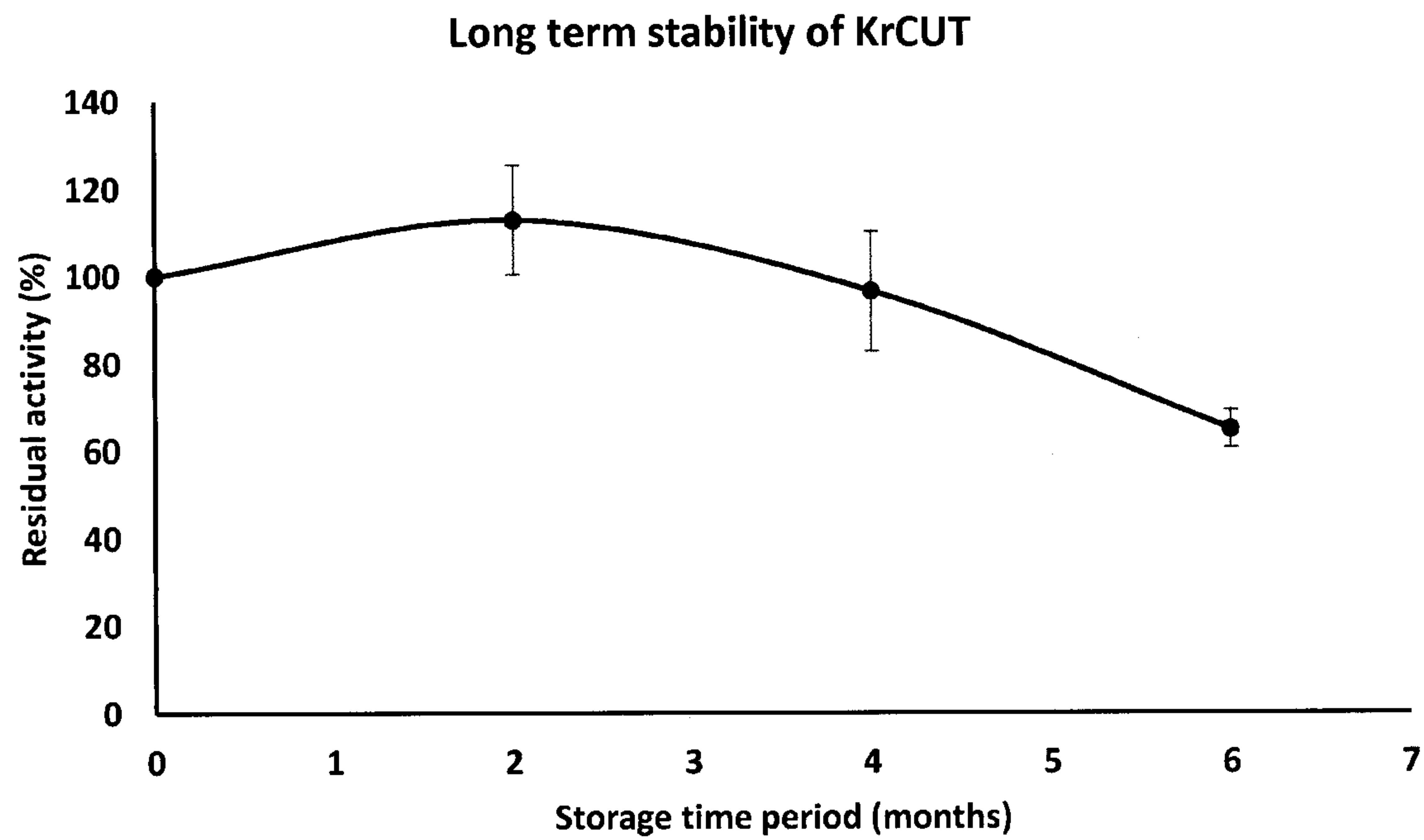


Figure 13

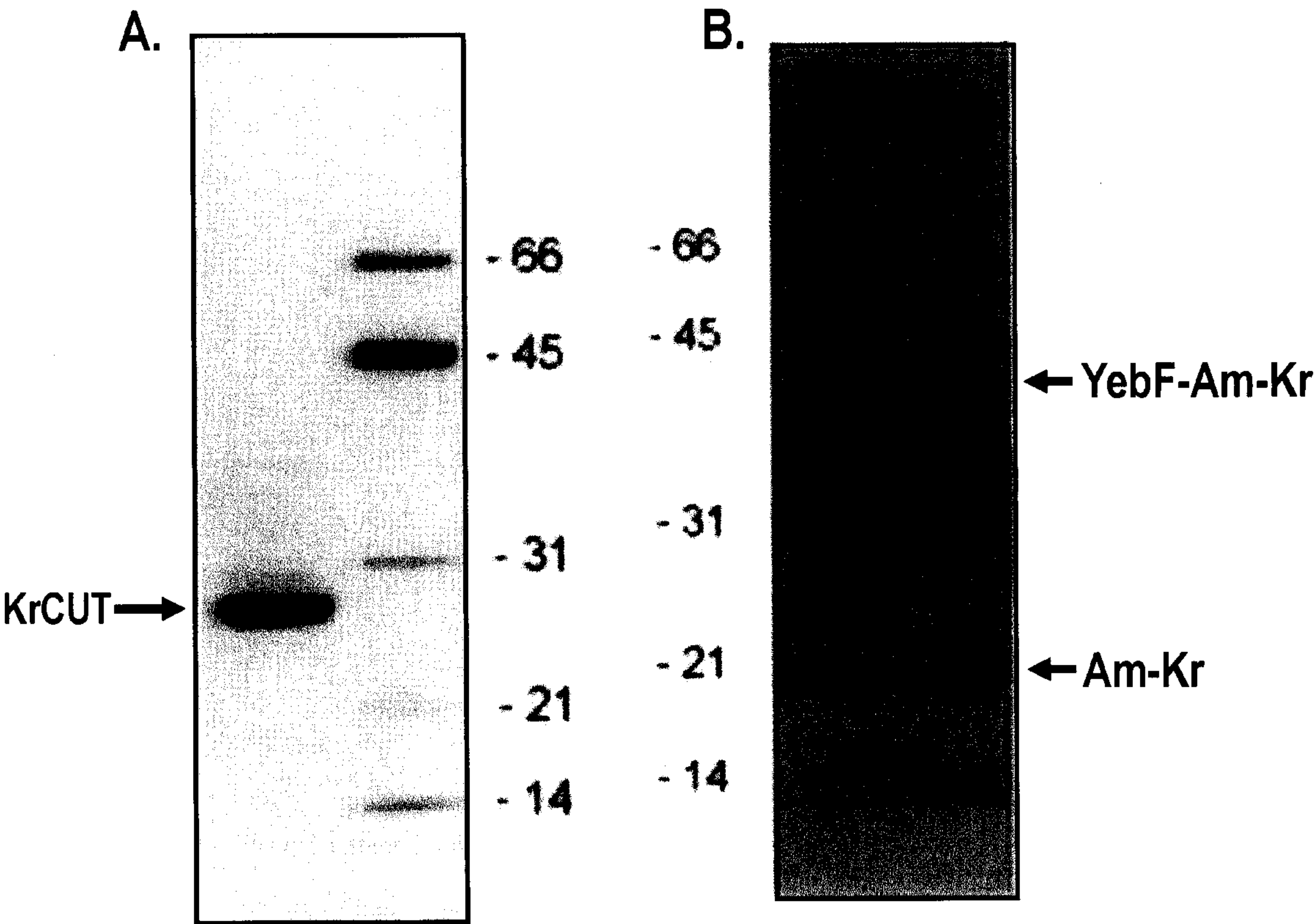


Figure 14

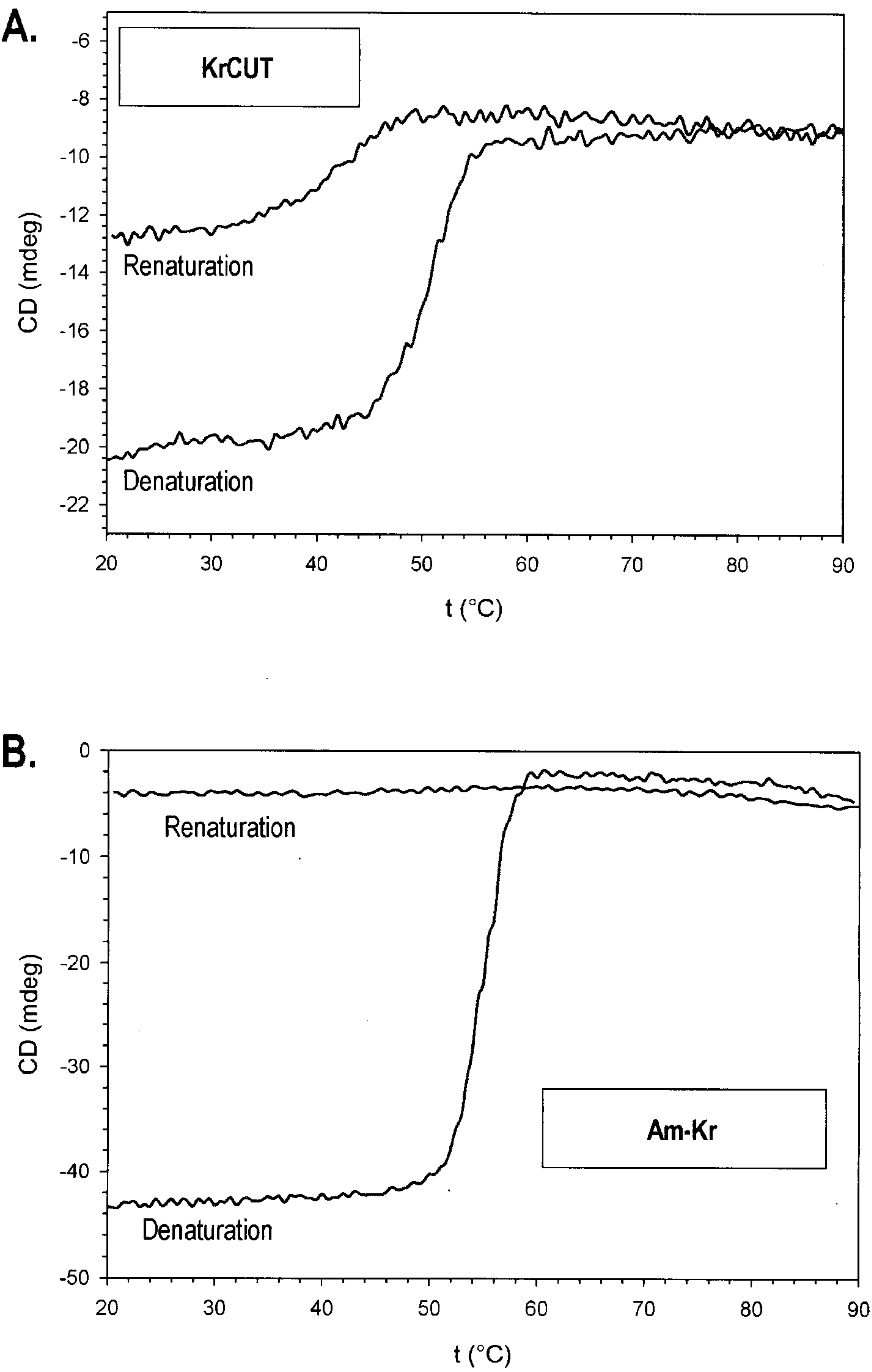


Figure 14

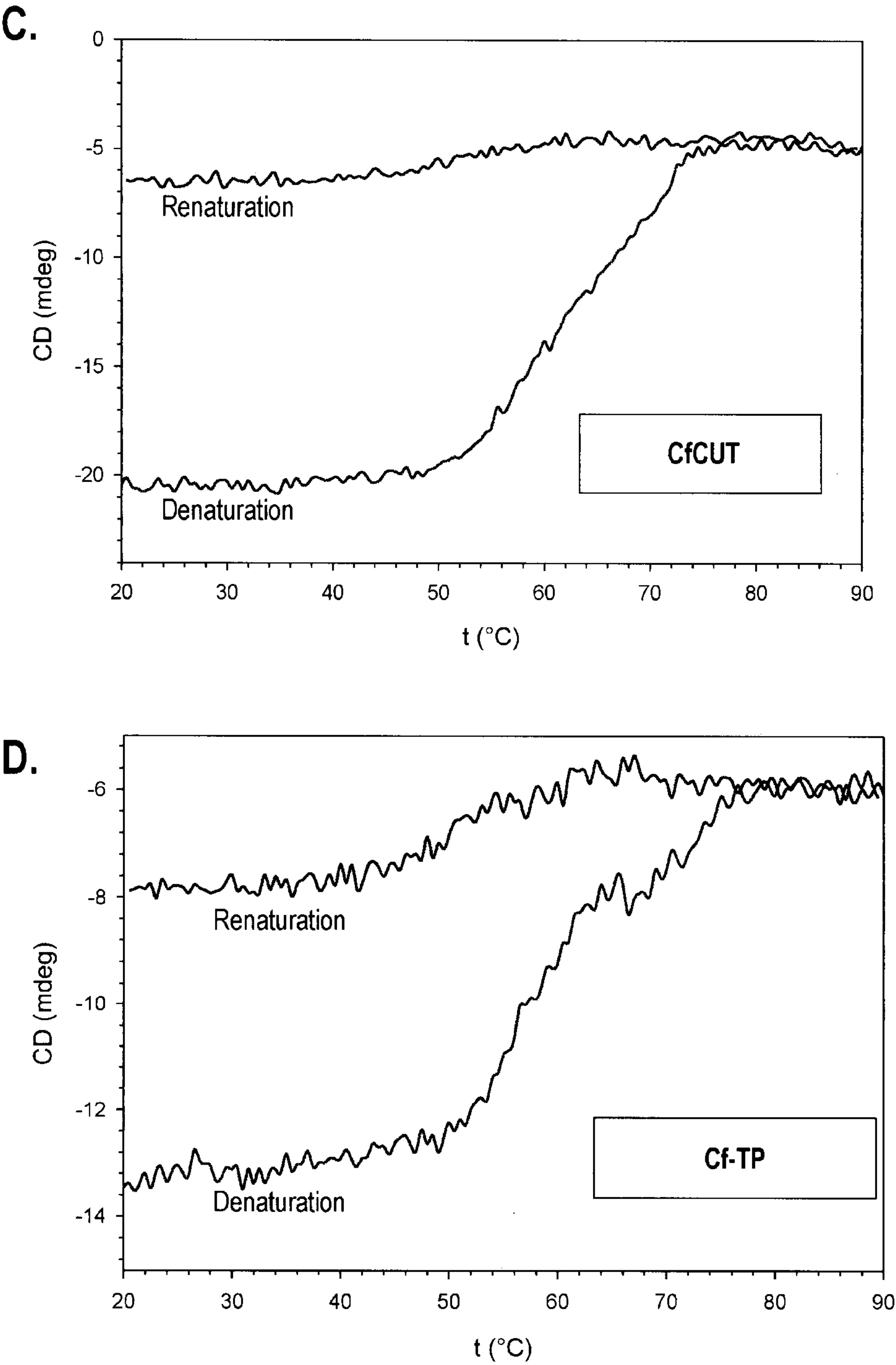


Figure 15

A.

CLUSTAL 2.1 multiple sequence alignment

KrCUT -ATCSDVDVVFARGTGETPGLGVGGPFVRSLTGELS DRTVTSHAVDYAASSSQASAGPG
CcCUT APACADVEVL FARGTGEAPGLGVLTGPFVRSVTSALSDRTVTSYAVNYAAESSQRSAGPG
CfCUT APACPDVELV FARGTGEAAGLGIVGRPLERALAAELPGRTVVATAVDYAASSSQASAGPG
CbCUT2 AGSCP DVQVVFARGTGERAGLGIIGRPFARALADELPGMTVTSHAVDYAAAASQRSAGPG
CbCUT1 TGDCPDVHVVFARGTGEPRGLGIVGRPFVSDLGDALPTMTVTSYAVNYSANASQTSAGPG
 ..*.:***** ***:.* *: *.* **.:**:*:* :** *****

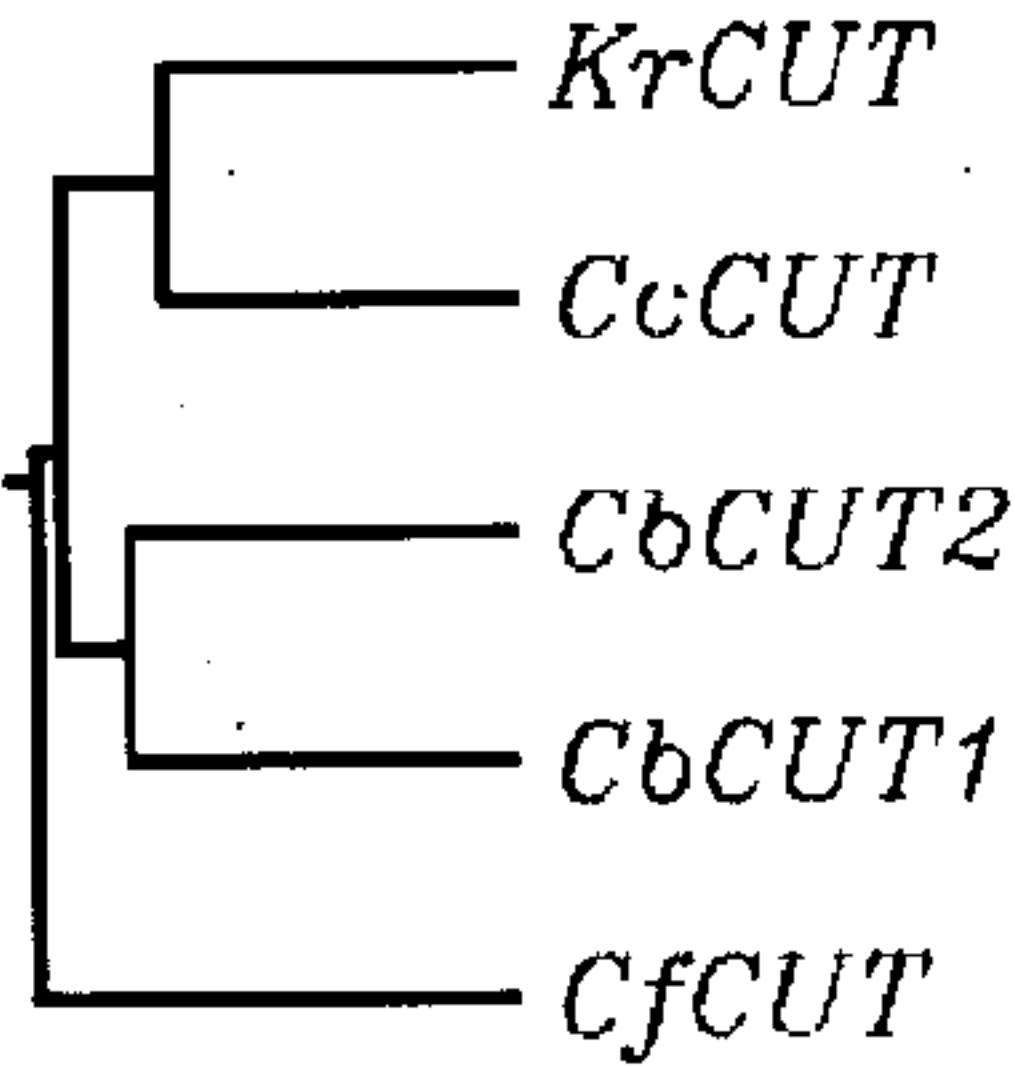
KrCUT ATAMSAHVREVAAC PSTRFVLGGYSQGATVTDIALGIRTGTTTGTPVPAELAGRVAAVV
CcCUT ATDLTNHLTATAAACPGTRFVLGGYSQGATVVDLALGIRTGTTTGTAIPAAL EPRVAAIV
CfCUT SGDMVAKVRSRAAACPGTQFVLGGYSQGATVTDLALGIRTGVTAGTALPEDLAARVAAVV
CbCUT2 ATAMTDHVTAMAARCPGTQFVLGGYSQGATVTSIALGIRAGTTTGRAIPDELSDRVAAVV
CbCUT1 AGDMTSHVTSMAARCPGTQFVLGGYSQGATVTSIAVGARSTSIRSRVLPANLEPRVAAVV
 : : .: ** **.*:*****.....*:.* *: .* * ****:*

KrCUT VFGNPLGLSGRTIATASSTYGPKSKDYCNSSDSVCGSAPKTGTGGHLSYASNGSTTDGAR
CcCUT VFGNPLGISGRTIATASPTYAARARDFCATGDPVCGGG--SSFAAHLAYRTNGDVTAGAD
CfCUT VYGNPLGLTRRTIAQAAPAFATRTVEYCNAGDPVCEPGG-GRFTHITYATNGTVLEGAR
CbCUT2 VFGNPLGMRGQTIASASRTYADRAKDYCN SGDSICGRQPSTGRGTHTGYATNGSTTDGAR
CbCUT1 VFGNPLGLTRRTIASEAPAYA AKSRDYCNRS DTVCGGR-GDARGGHLAYVSN GSVADGAA
 *:*****: :*** : :.: :.:* *.:* * * :** . **

KrCUT FAAGLVRAAGTPT-----TPTPTPTPTPVP-TTCVRDSTR
CcCUT FAAGLARATTVPVPTPTVPGTPTPSPTAPGTPTPVPTTSPTPAPSPTAPG-AACVRASTR
CfCUT FAAARVTAPAPT-----TPGPTGTPAPVPSAEPTPAPGDTCVTASSL
CbCUT2 FAAGLV TANPPEA-----APPTAPPTTPPTTPAPPSDQVTCVTAQVS
CbCUT1 FAASLVGVAP-----GDPASCVTARAV
 ***. . . : **

KrCUT DHVAADRAVSLYGRAYARGSRDSL GATSSYNV VSLQQVEGG-WRLVTAC
CcCUT AHVEAGRAERRHGIAYATGSGDRIGWVSSFVRVSVQQTADG-WERVLSC
CfCUT QHVRDGRAYPLWMRTYARGSGDPLGVLSSRTVVSLQADGDTWRKVAAC
CbCUT2 EHVEARRAIRGLTRAYARGTLEDIGRLRSTEEVSLRRSGTFSWTPTASC
CbCUT1 DHVEADRAFRSVL RAYARGSRDPLGRLTSSDLVSLQRTGQDSWSVVPAC
 ** ** : ** *: : :* * **.: * . :*

B.



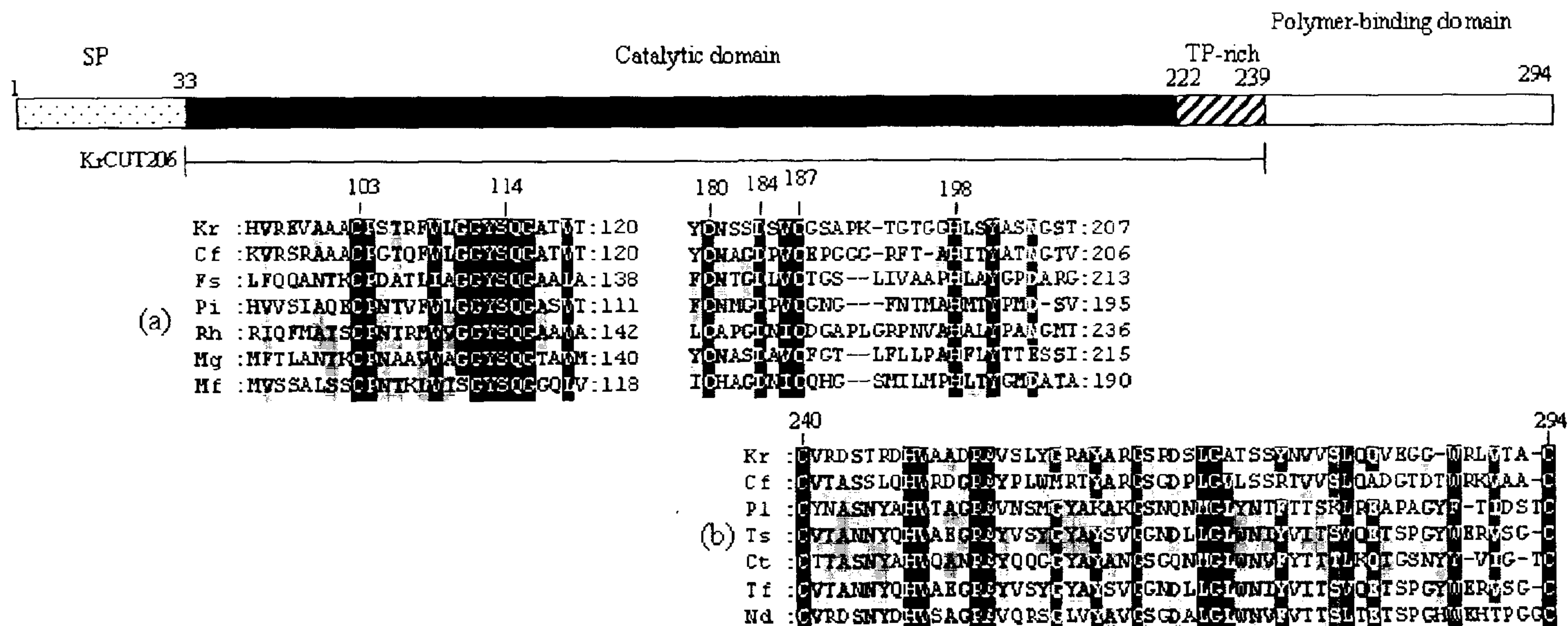


Figure 1