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<p>(54) Title: AMIDASE</p> <p>(57) Abstract</p> <p>A purified thermostable enzyme is derived from the archaeal bacterium <i>Thermococcus GUSL5</i>. The enzyme has a molecular weight of about 68.5 kilodaltons and has cellulase activity. The enzyme can be produced from native or recombinant host cells and can be used for the removal of arginine, phenylalanine, or methionine amino acids from the N-terminal end of peptides in peptide or peptidomimetic synthesis. The enzyme is selective for the L, or "natural" enantiomer of the amino acid derivatives and is therefore useful for the production of optically active compounds. These reactions can be performed in the presence of the chemically more reactive ester functionality, a step which is very difficult to achieve with nonenzymatic methods.</p>		

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### AMIDASE

This invention relates to newly identified polynucleotides, polypeptides encoded by such  
5 polynucleotides, the use of such polynucleotides and polypeptides, as well as the production and isolation of such polynucleotides and polypeptides. More particularly, the polypeptide of the present invention has been identified as an amidase and in particular an  
10 enzyme having activity in the removal of arginine, phenylalanine or methionine from the N-terminal end of peptides in peptide or peptidomimetic synthesis.

Thermophilic bacteria have received considerable attention as sources of highly active and thermostable  
15 enzymes (Bronneomeier, K. and Staudenbauer, W.L., D.R. Woods (Ed.), *The Clostridia and Biotechnology*, Butterworth Publishers, Stoneham, MA (1993). Recently, the most extremely thermophilic organotrophic eubacteria presently known have been isolated and characterized.  
20 These bacteria, which belong to the genus *Thermotoga*, are fermentative microorganisms metabolizing a variety of carbohydrates (Huber, R. and Stetter, K.O., in Ballows, et al., (Ed.), *The Procaryotes*, 2nd Ed., Springer-Verlag, New York, pgs. 3809-3819 (1992)).

25 Because to date most organisms identified from the archaeal domain are thermophiles or hyperthermophiles, archaeal bacteria are also considered a fertile source of thermophilic enzymes.

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SUMMARY OF THE INVENTION

In accordance with one aspect of the present invention, there is provided a novel enzyme, as well as active fragments, analogs and derivatives thereof.

5 In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules encoding an enzyme of the present invention including mRNAs, DNAs, cDNAs, genomic DNAs as well as active analogs and fragments of such enzymes.

10 In accordance with yet a further aspect of the present invention, there is provided a process for producing such polypeptide by recombinant techniques comprising culturing recombinant prokaryotic and/or eukaryotic host cells, containing a nucleic acid sequence  
15 encoding an enzyme of the present invention, under conditions promoting expression of said enzyme and subsequent recovery of said enzyme.

In accordance with yet a further aspect of the present invention, there is provided a process for  
20 utilizing such enzyme, or polynucleotide encoding such enzyme. The enzyme is useful for the removal of arginine, phenylalanine, or methionine amino acids from the N-terminal end of peptides in peptide or peptidomimetic synthesis. The enzyme is selective for  
25 the L, or "natural" enantiomer of the amino acid derivatives and is therefore useful for the production of optically active compounds. These reactions can be performed in the presence of the chemically more reactive ester functionality, a step which is very difficult to

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achieve with nonenzymatic methods. The enzyme is also able to tolerate high temperatures (at least 70°C), and high concentrations of organic solvents (>40% DMSO), both of which cause a disruption of secondary structure in peptides; this enables cleavage of otherwise resistant bonds.

In accordance with yet a further aspect of the present invention, there is also provided nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to a nucleic acid sequence of the present invention.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such enzymes, or polynucleotides encoding such enzymes, for *in vitro* purposes related to scientific research, for example, to generate probes for identifying similar sequences which might encode similar enzymes from other organisms.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

5           Figure 1 is an illustration of the full-length DNA and corresponding deduced amino acid sequence of the enzyme of the present invention. Sequencing was performed using a 378 automated DNA sequencer (Applied Biosystems, Inc.).

10           Figure 2 shows the fluorescence versus concentration of DMSO. The filled and open boxes represent individual assays from Example 3.

            Figure 3 shows the relative initial linear rates (increase in fluorescence per min. *i.e.* "activity")  
15 versus concentration of DMF for the more reactive CBZ-L-arg-AMC, from Example 3.

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DETAILED DESCRIPTION OF THE INVENTION

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

A coding sequence is "operably linked to" another coding sequence when RNA polymerase will transcribe the two coding sequences into a single mRNA, which is then translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the expressed sequences are ultimately processed to produce the desired protein.

"Recombinant" enzymes refer to enzymes produced by recombinant DNA techniques; *i.e.*, produced from cells transformed by an exogenous DNA construct encoding the desired enzyme. "Synthetic" enzymes are those prepared by chemical synthesis.

The present invention provides substantially pure amidase enzymes. The term "substantially pure" is used herein to describe a molecule, such as a polypeptide (*e.g.*, an amidase polypeptide, or a fragment thereof) that is substantially free of other proteins, lipids, carbohydrates, nucleic acids, and other biological materials with which it is naturally associated. For example, a substantially pure molecule, such as a polypeptide, can be at least 60%, by dry weight, the molecule of interest. The purity of the polypeptides can

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be determined using standard methods including, e.g., polyacrylamide gel electrophoresis (e.g., SDS-PAGE), column chromatography (e.g., high performance liquid chromatography (HPLC)), and amino-terminal amino acid  
5 sequence analysis.

A DNA "coding sequence of" or a "nucleotide sequence encoding" a particular enzyme, is a DNA sequence which is transcribed and translated into an enzyme when placed under the control of appropriate regulatory  
10 sequences. A "promotor sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The promoter is part of the DNA sequence. This sequence region has a start codon at its  
15 3' terminus. The promoter sequence does include the minimum number of bases where elements necessary to initiate transcription at levels detectable above background. However, after the RNA polymerase binds the sequence and transcription is initiated at the start  
20 codon (3' terminus with a promoter), transcription proceeds downstream in the 3' direction. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1) as well as protein binding domains  
25 (consensus sequences) responsible for the binding of RNA polymerase.

The present invention provides a purified thermostable enzyme that catalyzes the removal of arginine, phenylalanine, or methionine amino acids from  
30 the N-terminal end of peptides in peptide or peptidomimetic synthesis. The purified enzyme is an



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amidase derived from an organism referred to herein as  
"Thermococcus GU5L5" which is a thermophilic archaeal  
organism which has a very high temperature optimum. The  
organism is strictly anaerobic and grows between 55 and  
5 90°C (optimally at 85°C). GU5L5 was discovered in a  
shallow marine hydrothermal area in Vulcano, Italy. The  
organism has coccoid cells occurring in singlets or  
pairs. GU5L5 grows optimally at 85°C and pH 6.0 in a  
marine medium with peptone as a substrate and nitrogen in  
10 gas phase.

The polynucleotide of this invention was  
originally recovered from a genomic gene library derived  
from *Thermococcus* GU5L5 as described below. It contains  
an open reading frame encoding a protein of 622 amino  
15 acid residues.

In a preferred embodiment, the amidase enzyme of  
the present invention has a molecular weight of about  
68.5 kilodaltons as inferred from the nucleotide sequence  
of the gene.

20 In accordance with an aspect of the present  
invention, there are provided isolated nucleic acid  
molecules (polynucleotides) which encode for the mature  
enzyme having the deduced amino acid sequence of Figure 1  
(SEQ ID NO:2).

25 This invention, in addition to the isolated  
nucleic acid molecule encoding an amidase enzyme  
disclosed in Figure 1 (SEQ ID NO:1), also provides  
substantially similar sequences. Isolated nucleic acid  
sequences are substantially similar if: (i) they are

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capable of hybridizing under stringent conditions,  
hereinafter described, to SEQ ID NO:1; or (ii) they  
encode DNA sequences which are degenerate to SEQ ID NO:1.  
Degenerate DNA sequences encode the amino acid sequence  
5 of SEQ ID NO:2, but have variations in the nucleotide  
coding sequences. As used herein, "substantially  
similar" refers to the sequences having similar identity  
to the sequences of the instant invention. The  
nucleotide sequences that are substantially similar can  
10 be identified by hybridization or by sequence comparison.  
Enzyme sequences that are substantially similar can be  
identified by one or more of the following: proteolytic  
digestion, gel electrophoresis and/or microsequencing.

One means for isolating a nucleic acid molecule  
15 encoding an amidase enzyme is to probe a gene library  
with a natural or artificially designed probe using art  
recognized procedures (see, for example: Current  
Protocols in Molecular Biology, Ausubel F.M. et al.  
(EDS.) Green Publishing Company Assoc. and John Wiley  
20 Interscience, New York, 1989, 1992). It is appreciated  
to one skilled in the art that SEQ ID NO:1, or fragments  
thereof (comprising at least 15 contiguous nucleotides),  
is a particularly useful probe. Other particular useful  
probes for this purpose are hybridizable fragments to the  
25 sequences of SEQ ID NO:1 (i.e., comprising at least 15  
contiguous nucleotides).

With respect to nucleic acid sequences which  
hybridize to specific nucleic acid sequences disclosed  
herein, hybridization may be carried out under conditions  
30 of reduced stringency, medium stringency or even  
stringent conditions. As an example of oligonucleotide

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hybridization, a polymer membrane containing immobilized denatured nucleic acid is first prehybridized for 30 minutes at 45°C in a solution consisting of 0.9 M NaCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0, 5.0 mM Na<sub>2</sub>EDTA, 0.5% SDS, 10X Denhardt's, and 0.5 mg/mL polyriboadenylic acid. Approximately 2 X 10<sup>7</sup> cpm (specific activity 4-9 X 10<sup>8</sup> cpm/ug) of <sup>32</sup>P end-labeled oligonucleotide probe are then added to the solution. After 12-16 hours of incubation, the membrane is washed for 30 minutes at room temperature in 1X SET (150 mM NaCl, 20 mM Tris hydrochloride, pH 7.8, 1 mM Na<sub>2</sub>EDTA) containing 0.5% SDS, followed by a 30 minute wash in fresh 1X SET at T<sub>m</sub>-10°C for the oligo-nucleotide probe. The membrane is then exposed to auto-radiographic film for detection of hybridization signals.

Stringent conditions means hybridization will occur only if there is at least 90% identity, preferably at least 95% identity and most preferably at least 97% identity between the sequences. See J. Sambrook et al., Molecular Cloning, A Laboratory Manual (2d Ed. 1989) (Cold Spring Harbor Laboratory) which is hereby incorporated by reference in its entirety.

"Identity" as the term is used herein, refers to a polynucleotide sequence which comprises a percentage of the same bases as a reference polynucleotide (SEQ ID NO:1). For example, a polynucleotide which is at least 90% identical to a reference polynucleotide, has polynucleotide bases which are identical in 90% of the bases which make up the reference polynucleotide and may have different bases in 10% of the bases which comprise that polynucleotide sequence.

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The present invention also relates to polynucleotides which differ from the reference polynucleotide such that the changes are silent changes, for example the changes do not alter the amino acid  
5 sequence encoded by the polynucleotide. The present invention also relates to nucleotide changes which result in amino acid substitutions, additions, deletions, fusions and truncations in the enzyme encoded by the reference polynucleotide (SEQ ID NO:1). In a preferred  
10 aspect of the invention these enzymes retain the same biological action as the enzyme encoded by the reference polynucleotide.

It is also appreciated that such probes can be and are preferably labeled with an analytically detectable  
15 reagent to facilitate identification of the probe. Useful reagents include but are not limited to radioactivity, fluorescent dyes or enzymes capable of catalyzing the formation of a detectable product. The probes are thus useful to isolate complementary copies of  
20 DNA from other animal sources or to screen such sources for related sequences.

The coding sequence for the amidase enzyme of the present invention was identified by preparing a  
*Thermococcus* GU5L5 genomic DNA library and screening the  
25 library for the clones having amidase activity. Such methods for constructing a genomic gene library are well-known in the art. One means, for example, comprises shearing DNA isolated from GU5L5 by physical disruption. A small amount of the sheared DNA is checked on an  
30 agarose gel to verify that the majority of the DNA is in the desired size range (approximately 3-6 kb). The DNA

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is then blunt ended using Mung Bean Nuclease, incubated at 37°C and phenol/chloroform extracted. The DNA is then methylated using Eco RI Methylase. Eco R1 linkers are then ligated to the blunt ends through the use of T4 DNA  
5 ligase and incubation at 4°C. The ligation reaction is then terminated and the DNA is cut-back with Eco R1 restriction enzyme. The DNA is then size fractionated on a sucrose gradient following procedures known in the art, for example, Maniatis, T., et al., Molecular Cloning,  
10 Cold Spring Harbor Press, New York, 1982, which is hereby incorporated by reference in its entirety.

A plate assay is then performed to get an approximate concentration of the DNA. Ligation reactions are then performed and 1 µl of the ligation reaction is  
15 packaged to construct a library. Packaging, for example, may occur through the use of purified λgt11 phage arms cut with EcoRI and DNA cut with EcoRI after attaching EcoRI linkers. The DNA and λgt11 arms are ligated with DNA ligase. The ligated DNA is then packaged into  
20 infectious phage particles. The packaged phages are used to infect *E. coli* cultures and the infected cells are spread on agar plates to yield plates carrying thousands of individual phage plaques. The library is then amplified.

25 Fragments of the full length gene of the present invention may be used as a hybridization probe for a cDNA or a genomic library to isolate the full length DNA and to isolate other DNAs which have a high sequence similarity to the gene or similar biological activity.  
30 Probes of this type have at least 10, preferably at least 15, and even more preferably at least 30 bases and may

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contain, for example, at least 50 or more bases. The probe may also be used to identify a DNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete gene including 5 regulatory and promotor regions, exons, and introns.

The isolated nucleic acid sequences and other enzymes may then be measured for retention of biological activity characteristic to the enzyme of the present invention, for example, in an assay for detecting 10 enzymatic amidase activity. Such enzymes include truncated forms of amidase, and variants such as deletion and insertion variants.

The polynucleotide of the present invention may be in the form of DNA which DNA includes cDNA, genomic DNA, 15 and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature enzyme may be identical to the coding sequence shown in Figure 1 (SEQ ID NO:1) 20 and/or that of the deposited clone or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same mature enzyme as the DNA of Figure 1 (SEQ ID NO:1).

The polynucleotide which encodes for the mature 25 enzyme of Figure 1 (SEQ ID NO:2) may include, but is not limited to: only the coding sequence for the mature enzyme; the coding sequence for the mature enzyme and additional coding sequence such as a leader sequence or a proprotein sequence; the coding sequence for the mature 30 enzyme (and optionally additional coding sequence) and

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non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature enzyme.

Thus, the term "polynucleotide encoding an enzyme  
5 (protein)" encompasses a polynucleotide which includes only coding sequence for the enzyme as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants  
10 of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the enzyme having the deduced amino acid sequence of Figure 1 (SEQ ID NO:2). The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide  
15 or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature enzyme as shown in Figure 1 (SEQ ID NO:2) as well as variants of such  
20 polynucleotides which variants encode for a fragment, derivative or analog of the enzyme of Figure 1 (SEQ ID NO:2). Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

25 As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figure 1 (SEQ ID NO:1). As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which

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may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded enzyme.

The present invention also includes  
5 polynucleotides, wherein the coding sequence for the mature enzyme may be fused in the same reading frame to a polynucleotide sequence which aids in expression and secretion of an enzyme from a host cell, for example, a leader sequence which functions to control transport of  
10 an enzyme from the cell. The enzyme having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the enzyme. The polynucleotides may also encode for a proprotein which is the mature protein plus additional 5'  
15 amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

Thus, for example, the polynucleotide of the  
20 present invention may encode for a mature enzyme, or for an enzyme having a prosequence or for an enzyme having both a prosequence and a presequence (leader sequence).

The present invention further relates to polynucleotides which hybridize to the hereinabove-  
25 described sequences if there is at least 70%, preferably at least 90%, and more preferably at least 95% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described  
30 polynucleotides. As herein used, the term "stringent



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conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode enzymes which either retain substantially the same biological function or activity as the mature enzyme encoded by the DNA of Figure 1 (SEQ ID NO:1).

Alternatively, the polynucleotide may have at least 15 bases, preferably at least 30 bases, and more preferably at least 50 bases which hybridize to a polynucleotide of the present invention and which has an identity thereto, as hereinabove described, and which may or may not retain activity. For example, such polynucleotides may be employed as probes for the polynucleotide of SEQ ID NO:1, for example, for recovery of the polynucleotide or as a PCR primer.

Thus, the present invention is directed to polynucleotides having at least a 70% identity, preferably at least 90% identity and more preferably at least a 95% identity to a polynucleotide which encodes the enzyme of SEQ ID NO:2 as well as fragments thereof, which fragments have at least 30 bases and preferably at least 50 bases and to enzymes encoded by such polynucleotides.

The present invention further relates to a enzyme which has the deduced amino acid sequence of Figure 1 (SEQ ID NO:2), as well as fragments, analogs and derivatives of such enzyme.

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The terms "fragment," "derivative" and "analog" when referring to the enzyme of Figure 1 (SEQ ID NO:2) means a enzyme which retains essentially the same biological function or activity as such enzyme. Thus, an  
5 analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature enzyme.

The enzyme of the present invention may be a recombinant enzyme, a natural enzyme or a synthetic  
10 enzyme, preferably a recombinant enzyme.

The fragment, derivative or analog of the enzyme of Figure 1 (SEQ ID NO:2) may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably  
15 a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature enzyme is fused with  
20 another compound, such as a compound to increase the half-life of the enzyme (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature enzyme, such as a leader or secretory sequence or a sequence which is employed for  
25 purification of the mature enzyme or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

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The enzymes and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or enzyme present in a living animal is not isolated, but the same polynucleotide or enzyme, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or enzymes could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The enzymes of the present invention include the enzyme of SEQ ID NO:2 (in particular the mature enzyme) as well as enzymes which have at least 70% similarity (preferably at least 70% identity) to the enzyme of SEQ ID NO:2 and more preferably at least 90% similarity (more preferably at least 90% identity) to the enzyme of SEQ ID NO:2 and still more preferably at least 95% similarity (still more preferably at least 95% identity) to the enzyme of SEQ ID NO:2 and also include portions of such enzymes with such portion of the enzyme generally containing at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two enzymes is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one enzyme to the sequence of a second enzyme. Similarity

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may be determined by procedures which are well-known in the art, for example, a BLAST program (Basic Local Alignment Search Tool at the National Center for Biological Information).

5           A variant, i.e. a "fragment", "analog" or "derivative" enzyme, and reference enzyme may differ in amino acid sequence by one or more substitutions, additions, deletions, fusions and truncations, which may be present in any combination.

10           Among preferred variants are those that vary from a reference by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics. Typically seen as conservative  
15 substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the  
20 basic residues Lys and Arg and replacements among the aromatic residues Phe, Tyr.

Most highly preferred are variants which retain the same biological function and activity as the reference polypeptide from which it varies.

25           Fragments or portions of the enzymes of the present invention may be employed for producing the corresponding full-length enzyme by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length enzymes. Fragments or

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portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

The present invention also relates to vectors  
5 which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of enzymes of the invention by recombinant techniques.

Host cells are genetically engineered (transduced  
10 or transformed or transfected) with the vectors containing the polynucleotides of this invention. Such vectors may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc.  
15 The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the genes of the present invention. The culture conditions, such as temperature, pH and the like, are those  
20 previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing enzymes by recombinant  
25 techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing an enzyme. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA;  
30 baculovirus; yeast plasmids; vectors derived from

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combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

5           The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the  
10 scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be  
15 mentioned: LTR or SV40 promoter, the *E. coli*. *lac* or *trp*, the phage lambda P<sub>L</sub> promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation  
20 initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a  
25 phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*.

The vector containing the appropriate DNA sequence

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as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

5           As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli*, *Streptomyces*, *Bacillus subtilis*; fungal cells, such as yeast; insect cells such as *Drosophila S2* and *Spodoptera Sf9*; animal cells such as CHO, COS or Bowes melanoma;  
10 adenoviruses; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of  
15 the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further  
20 comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example;  
25 Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBluescript II (Stratagene); pTRC99a, pKK223-3, pDR540, pRIT2T (Pharmacia); Eukaryotic: pXT1, pSG5 (Stratagene) pSVK3, pBPV, pMSG, pSVLSV40 (Pharmacia). However, any other plasmid or vector may be used as long as they are  
30 replicable and viable in the host.

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Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named  
5 bacterial promoters include *lacI*, *lacZ*, T3, T7, *gpt*,  
*lambda P<sub>R</sub>*, *P<sub>L</sub>* and *trp*. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well  
10 within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic  
15 cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Dibner, M.,  
20 Battey, I., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the enzymes of the invention can be synthetically produced by  
25 conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs  
30 derived from the DNA constructs of the present invention.



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Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the enzymes of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about 10 from 10 to 300 bp that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and 15 adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* 20 TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK),  $\alpha$ -factor, acid phosphatase, or heat shock 25 proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated enzyme. Optionally, the 30 heterologous sequence can encode a fusion enzyme including an N-terminal identification peptide imparting

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desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence  
5 encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure  
10 maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas*, *Streptomyces*, and  
15 *Staphylococcus*, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication  
20 derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA).  
25 These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell  
30 density, the selected promoter is induced by appropriate

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means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the  
5 resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption,  
10 or use of cell lysing agents, such methods are well known to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of  
15 monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable  
20 promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites  
25 may be used to provide the required nontranscribed genetic elements.

The enzyme can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion

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or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can  
5 be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The enzymes of the present invention may be a  
10 naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host  
15 employed in a recombinant production procedure, the enzymes of the present invention may be glycosylated or may be non-glycosylated. Enzymes of the invention may or may not also include an initial methionine amino acid residue.

20 The enzymes, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes  
25 chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the enzymes  
30 corresponding to a sequence of the present invention can

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be obtained by direct injection of the enzymes into an animal or by administering the enzymes to an animal, preferably a nonhuman. The antibody so obtained will then bind the enzymes itself. In this manner, even a  
5 sequence encoding only a fragment of the enzymes can be used to generate antibodies binding the whole native enzymes. Such antibodies can then be used to isolate the enzyme from cells expressing that enzyme.

For preparation of monoclonal antibodies, any  
10 technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, *Nature*, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983,  
15 *Immunology Today* 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, et al., 1985, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single  
20 chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic enzyme products of this invention. Also, transgenic mice may be used to express humanized antibodies to immunogenic enzyme products of this invention.

25 Antibodies generated against the enzyme of the present invention may be used in screening for similar enzymes from other organisms and samples. Such screening techniques are known in the art, for example, one such screening assay is described in "Methods for Measuring  
30 Cellulase Activities", *Methods in Enzymology*, Vol 160,

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pp. 87-116, which is hereby incorporated by reference in its entirety. Antibodies may also be employed as a probe to screen gene libraries generated from this or other organisms to identify this or cross reactive activities.

5           The term "antibody," as used herein, refers to intact immunoglobulin molecules, as well as fragments of immunoglobulin molecules, such as Fab, Fab', (Fab')<sub>2</sub>, Fv, and SCA fragments, that are capable of binding to an epitope of an amidase polypeptide. These antibody  
10 fragments, which retain some ability to selectively bind to the antigen (e.g., an amidase antigen) of the antibody from which they are derived, can be made using well known methods in the art (see, e.g., Harlow and Lane, *supra*), and are described further, as follows.

15 (1) A Fab fragment consists of a monovalent antigen-binding fragment of an antibody molecule, and can be produced by digestion of a whole antibody molecule with the enzyme papain, to yield a fragment consisting of an intact light chain and a portion of a heavy chain.

20 (2) A Fab' fragment of an antibody molecule can be obtained by treating a whole antibody molecule with pepsin, followed by reduction, to yield a molecule consisting of an intact light chain and a portion of a heavy chain. Two Fab' fragments are obtained per  
25 antibody molecule treated in this manner.

(3) A (Fab')<sub>2</sub> fragment of an antibody can be obtained by treating a whole antibody molecule with the enzyme

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pepsin, without subsequent reduction. A (Fab')<sub>2</sub> fragment is a dimer of two Fab' fragments, held together by two disulfide bonds.

(4) An Fv fragment is defined as a genetically engineered fragment containing the variable region of a light chain and the variable region of a heavy chain expressed as two chains.

(5) A single chain antibody ("SCA") is a genetically engineered single chain molecule containing the variable region of a light chain and the variable region of a heavy chain, linked by a suitable, flexible polypeptide linker.

As used in this invention, the term "epitope" refers to an antigenic determinant on an antigen, such as an amidase polypeptide, to which the paratope of an antibody, such as an amidase-specific antibody, binds. Antigenic determinants usually consist of chemically active surface groupings of molecules, such as amino acids or sugar side chains, and can have specific three-dimensional structural characteristics, as well as specific charge characteristics.

The present invention is further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

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In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p  
5 preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In  
10 addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at  
15 certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 µg of  
20 plasmid or DNA fragment is used with about 2 units of enzyme in about 20 µl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 µg of DNA are digested with 20 to 250 units of enzyme in a larger volume.  
25 Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is  
30 electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.



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Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. et al., *Nucleic Acids Res.*, 8:4057 (1980).

"Oligonucleotides" refers to either a single  
5 stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides may or may not have a 5' phosphate. Those that do not will not ligate to another oligonucleotide without adding a  
10 phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic  
15 acid fragments (Maniatis et al., *Id.*, p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 µg of approximately equimolar amounts of the DNA fragments to be ligated.

20 Unless otherwise stated, transformation was performed as described in the method of Sambrook, Fritsch and Maniatus, 1989.

#### Example 1

##### Bacterial Expression and Purification of Amidase

25 A *Thermococcus* GU5L5 genomic library was screened for amidase activity as described in Example 2 and a positive clone was identified and isolated. DNA of this clone was used as a template in a 100 µl PCR reaction using the following primer sequences:

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5' primer: CCGAGAATTC ATTAAGAGG AGAAATTAAC TATGACCGGC  
ATCGAATGGA 3' (SEQ ID NO:3). 3' primer: 5' AATAAGGATC  
CACACTGGCA CAGTGTCAAG ACA 3' (SEQ ID NO:4).

The protein was expressed in *E. coli*. The gene  
5 was amplified using PCR with the primers indicated above.

Subsequent to amplification, the PCR product was  
cloned into the *EcoRI* and *BamHI* sites of pQET1 and  
transformed by electroporation into *E. coli* M15(pREP4).  
The resulting transformants were grown up in 3ml  
10 cultures, and a portion of this culture was induced. A  
portion of the uninduced and induced cultures were  
assayed using Z-L-Phe-AMC (see below).

The primer sequences set out above may also be  
employed to isolate the target gene from the deposited  
15 material by hybridization techniques described above.

### Example 2

#### Discovery of an amidase from *Thermococcus* GU5L5

##### Production of the expression gene bank.

Colonies containing pBluescript plasmids with  
20 random inserts from the organism *Thermococcus* GU5L5 was  
obtained according to the method of Hay and Short. (Hay,  
B. and Short, J., *Strategies*. 1992, 5, 16.) The  
resulting colonies were picked with sterile toothpicks  
and used to singly inoculate each of the wells of 96-well  
25 microtiter plates. The wells contained 250  $\mu$ L of LB  
media with 100  $\mu$ g/mL ampicillin, 80  $\mu$ g/mL methicillin,  
and 10% v/v glycerol (LB Amp/Meth, glycerol). The cells  
were grown overnight at 37°C without shaking. This

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constituted generation of the "SourceGeneBank"; each well of the Source GeneBank thus contained a stock culture of *E. coli* cells, each of which contained a pBluescript plasmid with a unique DNA insert.

#### 5 Screening for amidase activity.

The plates of the Source GeneBank were used to multiply inoculate a single plate (the "Condensed Plate") containing in each well 200  $\mu$ L of LB Amp/Meth, glycerol. This step was performed using the High Density  
10 Replicating Tool (HDRT) of the Beckman Biomek with a 1% bleach, water, isopropanol, air-dry sterilization cycle in between each inoculation. Each well of the Condensed Plate thus contained 10 to 12 different pBluescript clones from each of the source library plates. The  
15 Condensed Plate was grown for 16h at 37°C and then used to inoculate two white 96-well Polyfiltronics microtiter daughter plates containing in each well 250  $\mu$ L of LB Amp/Meth (without glycerol). The original condensed plate was put in storage -80°C. The two condensed  
20 daughter plates were incubated at 37°C for 18 h.

The '600  $\mu$ M substrate stock solution' was prepared as follows: 25 mg of N-morphourea-L-phenylalanyl-7-amido-4-trifluoromethylcoumarin (Mu-Phe-AFC, Enzyme Systems Products, Dublin, CA) was dissolved in the  
25 appropriate volume of DMSO to yield a 25.2 mM solution. Two hundred fifty microliters of DMSO solution was added to ca. 9 mL of 50 mM, pH 7.5 Hepes buffer containing 0.6 mg/mL of dodecyl maltoside. The volume was taken to 10.5 mL with the above Hepes buffer to yield a cloudy  
30 solution.

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**Mu-Phe-AFC**

Fifty  $\mu\text{L}$  of the '600  $\mu\text{M}$  stock solution' was added to each of the wells of a white condensed plate using the Biomek to yield a final concentration of substrate of  
5 ~100  $\mu\text{M}$ . The fluorescence values were recorded (excitation = 400 nm, emission = 505 nm) on a plate reading fluorometer immediately after addition of the substrate. The plate was incubated at 70°C for 60 min. and the fluorescence values were recorded again. The  
10 initial and final fluorescence values were subtracted to determine if an active clone was present by an increase in fluorescence over the majority of the other wells.

**Isolation of the active clone.**

In order to isolate the individual clone which  
15 carried the activity, the Source GeneBank plates were thawed and the individual wells used to singly inoculate a new plate containing LB Amp/Meth. As above the plate was incubated at 37°C to grow the cells, and 50  $\mu\text{L}$  of 600  $\mu\text{M}$  substrate stock solution added using the Biomek. Once  
20 the active well from the source plate was identified, the cells from the source plate were used to inoculate 3mL cultures of LB/AMP/Meth, which were grown overnight. The plasmid DNA was isolated from the cultures and utilized for sequencing and construction of expression subclones.

25

**Example 3****Thermococcus GU5L5 Amidase characterization****Substrate specificity.**

Using the following substrates (see below for definitions of the abbreviations): CBZ-L-ala-AMC, CBZ-L-

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arg-AMC, CBZ-L-met-AMC, CBZ-L-phe-AMC, and 7-methyl-umbelliferyl heptanoate at 100 $\mu$ M for 1 hour at 70°C in the assays as described in the clone discovery section, the relative activity of the amidase was 3:3:1:<0.1: <0.1  
5 for the compounds CBZ-L-arg-AMC : CBZ-L-phe-AMC : CBZ-L-met-AMC : CBZ-L-ala-AMC : 7-methylumbelliferyl heptanoate. The excitation and emission wavelengths for the 7-amido-4-methylcoumarins were 380 and 460 nm respectively, and 326 and 450 for the  
10 methylumbelliferone.

The abbreviations stand for the following compounds:

CBZ-L-ala-AMC = N $\alpha$ -carbonylbenzyloxy-L-alanine-7-amido-4-methylcoumarin

15 CBZ-L-arg-AMC = N $\alpha$ -carbonylbenzyloxy-L-arginine-7-amido-4-methylcoumarin

CBZ-D-arg-AMC = N $\alpha$ -carbonylbenzyloxy-D-arginine-7-amido-4-methylcoumarin

20 CBZ-L-met-AMC = N $\alpha$ -carbonylbenzyloxy-L-methionine-7-amido-4-methylcoumarin

CBZ-L-phe-AMC = N $\alpha$ -carbonylbenzyloxy-L-phenylalanine-7-amido-4-methylcoumarin

#### **Organic solvent sensitivity.**

The activity of the amidase in increasing  
25 concentrations of dimethyl sulfoxide (DMSO) was tested as follows: to each well of a microtiter plate was added 10  $\mu$ L of 3 mM CBZ-L-phe-AMC in DMSO, 25  $\mu$ L of cell lysate containing the amidase activity, and 250  $\mu$ L of a variable mixture of DMSO:pH 7.5, 50 mM Hepes buffer. The  
30 reactions were heated for 1 hour at 70°C and the

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fluorescence measured. Figure 2 shows the fluorescence versus concentration of DMSO. The filled and open boxes represent individual assays.

The activity and enantioselectivity of the amidase  
 5 in increasing concentrations of dimethyl formamide (DMF)  
 was tested as follows: to each well of a microtiter  
 plate was added 30  $\mu$ L of 1 mM CBZ-L-arg-AMC or CBZ-D-arg-  
 AMC in DMF, 30  $\mu$ L of cell lysate containing the amidase  
 activity, and 240  $\mu$ L of a variable mixture of DMF:pH 7.5,  
 10 50 mM Hepes buffer. The reactions were incubated at RT  
 for 1 hour and the fluorescence measured at 1 minute  
 intervals. Figure 3 shows the relative initial linear  
 rates (increase in fluorescence per min, i.e.,  
 'activity') versus concentration of DMF for the more  
 15 reactive CBZ-L-arg-AMC.

The initial linear rate ('activity') of the L and  
 the D CBZ-arg-AMC substrates are shown in Tables 1 and 2  
 below:

Table 1

20 Activity of the CBZ-L-  
 arg-AMC:

DMF	Initial Rate, Fl.U./min
0.4%	654
10%	2548
20%	1451
30%	541
40%	345

Table 2

Activity of the CBZ-D-  
 arg-AMC:

DMF	Initial Rate, Fl.U./min
0.4%	0.3
10%	10.1
20%	4.6
30%	1.8
40%	0.9

50%	303
60%	190
75%	81
90%	11

50%	1.2
60%	1.4
75%	0.1
90%	0.1

5           The above data indicate that the enzyme shows excellent selectivity for the L, or 'natural' enantiomer of the derivatized amino acid substrate.

          Numerous modifications and variations of the present invention are possible in light of the above  
10 teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.

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## SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- (i) APPLICANT: Recombinant Biocatalysis, Inc.
- (ii) TITLE OF INVENTION: Amidases
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: FISH & RICHARDSON  
(B) STREET: 4225 EXECUTIVE SQUARE, STE. 1400  
(C) CITY: LA JOLLA  
(D) STATE: CA  
(E) COUNTRY: USA  
(F) ZIP: 92037
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: 3.5 INCH DISKETTE  
(B) COMPUTER: IBM PS/2  
(C) OPERATING SYSTEM: MS-DOS  
(D) SOFTWARE: WORD PERFECT 6.0
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER: Unassigned  
(B) FILING DATE: Herewith  
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: 08/664,646  
(B) FILING DATE: 17 June 1996
- (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: LISA A. HAILE, Ph.D.  
(B) REGISTRATION NUMBER: 38,347  
(C) REFERENCE/DOCKET NUMBER: 09010/005W01
- (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: 619-678-5070  
(B) TELEFAX: 619-678-5099



(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 1869 NUCLEOTIDES
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATG ACC GGC ATC GAA TGG AAC CAC GAG ACC TTT TCT AAG TTC GCC TAC	48
Met Thr Gly Ile Glu Trp Asn His Glu Thr Phe Ser Lys Phe Ala Tyr	
5 10 15	
CTG GGC GAC CCG AGG ATA CGG GGA AAC TTA ATC GCG TAC ACC CTG ACG	96
Leu Gly Asp Pro Arg Ile Arg Gly Asn Leu Ile Ala Tyr Thr Leu Thr	
20 25 30	
AAG GCC AAC ATG AAG GAC AAC AAG TAC GAG AGC ACG GTT GTT GTT GAA	144
Lys Ala Asn Met Lys Asp Asn Lys Tyr Glu Ser Thr Val Val Val Glu	
35 40 45	
GAC CTT GAA ACG GGC TCA AGG CGC TTC ATC GAG AAC GCC TCA ATG CCG	192
Asp Leu Glu Thr Gly Ser Arg Arg Phe Ile Glu Asn Ala Ser Met Pro	
50 55 60	
AGG ATT TCG CCA GAC GGC AGA AAG CTC GCC TTC ACC TGC TTT AAC GAG	240
Arg Ile Ser Pro Asp Gly Arg Lys Leu Ala Phe Thr Cys Phe Asn Glu	
65 70 75 80	
GAG AAG AAG GAG ACC GAG ATA TGG GTG GCC GAT ATC CAG ACC CTG AGC	288
Glu Lys Lys Glu Thr Glu Ile Trp Val Ala Asp Ile Gln Thr Leu Ser	
85 90 95	
GCC AAG AAA GTC CTC TCA ACT AAA AAC GTC CGC TCG ATG CAG TGG AAC	336
Ala Lys Lys Val Leu Ser Thr Lys Asn Val Arg Ser Met Gln Trp Asn	
100 105 110	
GAC GAT TCA AGG AGA CTC TTA GTT GTC GGC TTC AAG AGG AGG GAC GAT	384
Asp Asp Ser Arg Arg Leu Leu Val Val Gly Phe Lys Arg Arg Asp Asp	
115 120 125	
GAG GAC TTC GTC TTT GAC GAC GAC GTC CCG GTC TGG TTC GAC AAT ATG	432
Glu Asp Phe Val Phe Asp Asp Asp Val Pro Val Trp Phe Asp Asn Met	
130 135 140	
GGA TTC TTT GAT GGA GAG AAG ACG ACG TTC TGG GTT CTT GAC ACT GAG	480
Gly Phe Phe Asp Gly Glu Lys Thr Thr Phe Trp Val Leu Asp Thr Glu	
145 150 155 160	
GCC GAG GAG ATA ATC GAG CAG TTC GAG AAG CCG AGG TTT TCG AGT GGC	528
Ala Glu Glu Ile Ile Glu Gln Phe Glu Lys Pro Arg Phe Ser Ser Gly	
165 170 175	
CTC TGG CAC GGC GAT GCG ATA GTT GTG AAC GTC CCG CAC CGC GAG GGG	576
Leu Trp His Gly Asp Ala Ile Val Val Asn Val Pro His Arg Glu Gly	
180 185 190	

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AGC Ser	AAG Lys	CCT Pro	GCC Ala	CTG Leu	TTC Phe	AAG Lys	TTC Phe	TAC Tyr	GAC Asp	ATA Ile	GTC Val	CTA Leu	TGG Trp	AAG Lys	GAC Asp	624
		195					200					205				
GGG Gly	GAG Glu	GAA Glu	GAG Glu	AAG Lys	CTC Leu	TTC Phe	GAG Glu	AGG Arg	GTC Val	TCC Ser	TTC Phe	GAG Glu	GCG Ala	GTT Val	GAC Asp	672
	210					215					220					
TCC Ser	GAC Asp	GGA Gly	AAG Lys	AGA Arg	ATA Ile	CTC Leu	CTG Leu	AGG Arg	GGC Gly	AAG Lys	AAA Lys	AAA Lys	AAG Lys	CGG Arg	TTC Phe	720
	225				230					235					240	
ATC Ile	AGC Ser	GAG Glu	CAC His	GAC Asp	TGG Trp	CTG Leu	TAC Tyr	CTC Leu	TGG Trp	GAC Asp	GGC Gly	GAG Glu	CTT Leu	AAA Lys	CCG Pro	768
				245					250					255		
ATC Ile	TAC Tyr	GAG Glu	GGC Gly	CCG Pro	CTC Leu	GAC Asp	GTC Val	TGG Trp	GAA Glu	GCC Ala	AAG Lys	CTC Leu	ACG Thr	GAA Glu	GGA Gly	816
			260				265						270			
AAG Lys	GTC Val	TAC Tyr	TTC Phe	CTC Leu	ACT Thr	CCA Pro	GAT Asp	GCG Ala	GGC Gly	AGG Arg	GTA Val	AAC Asn	CTC Leu	TGG Trp	CTC Leu	864
		275					280					285				
TGG Trp	GAC Asp	GGG Gly	AAG Lys	GCC Ala	GAG Glu	CGT Arg	GTT Val	GTT Val	ACC Thr	GGC Gly	GAC Asp	CAC His	TGG Trp	ATT Ile	TAC Tyr	912
	290					295					300					
GGG Gly	CTT Leu	GAC Asp	GTC Val	AGC Ser	GAT Asp	GGC Gly	AAA Lys	GCA Ala	TTG Leu	CTC Leu	CTC Leu	ATC Ile	ATG Met	ACC Thr	GCC Ala	960
	305				310					315					320	
ACG Thr	AGG Arg	ATA Ile	GGC Gly	GAG Glu	CTC Leu	TAC Tyr	CTC Leu	TAC Tyr	GAC Asp	GGC Gly	GAG Glu	CTG Leu	AAA Lys	CAG Gln	GTC Val	1008
				325					330					335		
ACC Thr	GAA Glu	TAC Tyr	AAC Asn	GGG Gly	CCG Pro	ATA Ile	TTC Phe	AGG Arg	AAG Lys	CTC Leu	AAG Lys	ACC Thr	TTC Phe	GAG Glu	CCG Pro	1056
			340					345					350			
AGG Arg	CAC His	TTC Phe	CGC Arg	TTC Phe	AAG Lys	AGC Ser	AAA Lys	GAC Asp	CTC Leu	GAG Glu	ATA Ile	GAC Asp	GGC Gly	TGG Trp	TAC Tyr	1104
		355					360					365				
CTC Leu	AGG Arg	CCG Pro	GAG Glu	GTT Val	AAA Lys	GAG Glu	GAG Glu	AAG Lys	GCC Ala	CCG Pro	GTG Val	ATA Ile	GTC Val	TTC Phe	GTC Val	1152
	370					375					380					
CAC His	GGC Gly	GGG Gly	CCG Pro	AAG Lys	GGC Gly	ATG Met	TAC Tyr	GGA Gly	CAC His	CGC Arg	TTC Phe	GTC Val	TAC Tyr	GAG Glu	ATG Met	1200
	385				390					395				400		
CAG Gln	CTG Leu	ATG Met	GCG Ala	AGC Ser	AAG Lys	GGC Gly	TAC Tyr	TAC Tyr	TGC Val	TGC Val	TTC Phe	GTG Val	AAC Asn	CCG Pro	CGC Arg	1248
				405					410					415		
GGC Gly	AGC Ser	GAC Asp	GGC Gly	TAT Tyr	AGC Ser	GAA Glu	GAC Asp	TTC Phe	GCG Ala	CTC Leu	CGC Arg	GTC Val	CTG Leu	GAG Glu	AGG Arg	1296
			420					425					430			

ACT	GGC	TTG	GAG	GAC	TTT	GAG	GAC	ATA	ATG	AAC	GGC	ATC	GAG	GAG	TTC	1344
Thr	Gly	Leu	Glu	Asp	Phe	Glu	Asp	Ile	Met	Asn	Gly	Ile	Glu	Glu	Phe	
		435					440					445				
TTC	AAG	CTC	GAA	CCG	CAG	GCC	GAC	AGG	GAG	CGC	GTT	GGA	ATA	ACG	GGC	1392
Phe	Lys	Leu	Glu	Pro	Gln	Ala	Asp	Arg	Glu	Arg	Val	Gly	Ile	Thr	Gly	
	450					455					460					
ATA	AGC	TAC	GGC	GGC	TTC	ATG	ACC	AAC	TGG	GCC	TTG	ACT	CAG	AGC	GAC	1440
Ile	Ser	Tyr	Gly	Gly	Phe	Met	Thr	Asn	Trp	Ala	Leu	Thr	Gln	Ser	Asp	
465					470					475					480	
CTC	TTC	AAG	GCA	GGA	ATA	AGC	GAG	AAC	GGC	ATA	AGC	TAC	TGG	CTC	ACC	1488
Leu	Phe	Lys	Ala	Gly	Ile	Ser	Glu	Asn	Gly	Ile	Ser	Tyr	Trp	Leu	Thr	
				485					490					495		
AGC	TAC	GCC	TTC	TCG	GAC	ATA	GGG	CTC	TGG	TAC	GAC	GTC	GAG	GTC	ATC	1536
Ser	Tyr	Ala	Phe	Ser	Asp	Ile	Gly	Leu	Trp	Tyr	Asp	Val	Glu	Val	Ile	
			500					505					510			
GGG	CCA	AAT	CCG	TTA	GAG	AAC	GAG	AAC	TTC	AGG	AAG	CTC	AGC	CCG	CTG	1584
Gly	Pro	Asn	Pro	Leu	Glu	Asn	Glu	Asn	Phe	Arg	Lys	Leu	Ser	Pro	Leu	
		515					520					525				
TTC	TAC	GCT	CAG	AAC	GTG	AAG	GCG	CCG	ATA	CTC	CTA	ATC	CAC	TCG	CTT	1632
Phe	Tyr	Ala	Gln	Asn	Val	Lys	Ala	Pro	Ile	Leu	Leu	Ile	His	Ser	Leu	
	530					535						540				
GAG	GAC	TAC	CGC	TGT	CCG	CTC	GAC	CAG	AGC	CTT	ATG	TTC	TAC	AAC	GTG	1680
Glu	Asp	Tyr	Arg	Cys	Pro	Leu	Asp	Gln	Ser	Leu	Met	Phe	Tyr	Asn	Val	
545					550					555					560	
CTC	AAG	GAC	ATG	GGC	AAG	GAA	GCC	TAC	ATA	GCG	ATA	TTC	AAG	CGC	GGC	1728
Leu	Lys	Asp	Met	Gly	Lys	Glu	Ala	Tyr	Ile	Ala	Ile	Phe	Lys	Arg	Gly	
				565					570					575		
GCC	CAC	GGC	CAC	AGC	GTC	CGC	GGA	AGC	CCG	AGG	CAC	AGG	CCG	AAG	CGC	1776
Ala	His	Gly	His	Ser	Val	Arg	Gly	Ser	Pro	Arg	His	Arg	Pro	Lys	Arg	
			580					585					590			
TAC	AGG	CTC	TTC	ATA	GAG	TTC	TTC	GAG	CGC	AAG	CTC	AAG	AAG	TAC	GAG	1824
Tyr	Arg	Leu	Phe	Ile	Glu	Phe	Phe	Glu	Arg	Lys	Leu	Lys	Lys	Tyr	Glu	
		595					600						605			
GAG	GGC	TTT	GAG	GTA	GAG	AAG	ATA	CTC	AAG	GGG	AAT	GGG	AAC	TGA		1869
Glu	Gly	Phe	Glu	Val	Glu	Lys	Ile	Leu	Lys	Gly	Asn	Gly	Asn			
	610					615					620					

- (2) INFORMATION FOR SEQ ID NO:2:
- (i) SEQUENCE CHARACTERISTICS
    - (A) LENGTH: 622 AMINO ACIDS
    - (B) TYPE: AMINO ACID
    - (C) STRANDEDNESS:
    - (D) TOPOLOGY: LINEAR
  - (ii) MOLECULE TYPE: PROTEIN

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Thr Gly Ile Glu Trp Asn His Glu Thr Phe Ser Lys Phe Ala Tyr  
5 10 15

Leu Gly Asp Pro Arg Ile Arg Gly Asn Leu Ile Ala Tyr Thr Leu Thr  
20 25 30

Lys Ala Asn Met Lys Asp Asn Lys Tyr Glu Ser Thr Val Val Val Glu  
35 40 45

Asp Leu Glu Thr Gly Ser Arg Arg Phe Ile Glu Asn Ala Ser Met Pro  
50 55 60

Arg Ile Ser Pro Asp Gly Arg Lys Leu Ala Phe Thr Cys Phe Asn Glu  
65 70 75 80

Glu Lys Lys Glu Thr Glu Ile Trp Val Ala Asp Ile Gln Thr Leu Ser  
85 90 95

Ala Lys Lys Val Leu Ser Thr Lys Asn Val Arg Ser Met Gln Trp Asn  
100 105 110

Asp Asp Ser Arg Arg Leu Leu Val Val Gly Phe Lys Arg Arg Asp Asp  
115 120 125

Glu Asp Phe Val Phe Asp Asp Asp Val Pro Val Trp Phe Asp Asn Met  
130 135 140

Gly Phe Phe Asp Gly Glu Lys Thr Thr Phe Trp Val Leu Asp Thr Glu  
145 150 155 160

Ala Glu Glu Ile Ile Glu Gln Phe Glu Lys Pro Arg Phe Ser Ser Gly  
165 170 175

Leu Trp His Gly Asp Ala Ile Val Val Asn Val Pro His Arg Glu Gly  
180 185 190

Ser Lys Pro Ala Leu Phe Lys Phe Tyr Asp Ile Val Leu Trp Lys Asp  
195 200 205

Gly Glu Glu Glu Lys Leu Phe Glu Arg Val Ser Phe Glu Ala Val Asp  
210 215 220

Ser Asp Gly Lys Arg Ile Leu Leu Arg Gly Lys Lys Lys Arg Phe  
225 230 235 240

Ile Ser Glu His Asp Trp Leu Tyr Leu Trp Asp Gly Glu Leu Lys Pro  
245 250 255

Ile Tyr Glu Gly Pro Leu Asp Val Trp Glu Ala Lys Leu Thr Glu Gly  
260 265 270

Lys Val Tyr Phe Leu Thr Pro Asp Ala Gly Arg Val Asn Leu Trp Leu  
275 280 285

Trp Asp Gly Lys Ala Glu Arg Val Val Thr Gly Asp His Trp Ile Tyr  
290 295 300

Gly Leu Asp Val Ser Asp Gly Lys Ala Leu Leu Leu Ile Met Thr Ala  
305 310 315 320

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Thr Arg Ile Gly Glu Leu Tyr Leu Tyr Asp Gly Glu Leu Lys Gln Val  
 325 330 335

Thr Glu Tyr Asn Gly Pro Ile Phe Arg Lys Leu Lys Thr Phe Glu Pro  
 340 345 350

Arg His Phe Arg Phe Lys Ser Lys Asp Leu Glu Ile Asp Gly Trp Tyr  
 355 360 365

Leu Arg Pro Glu Val Lys Glu Glu Lys Ala Pro Val Ile Val Phe Val  
 370 375 380

His Gly Gly Pro Lys Gly Met Tyr Gly His Arg Phe Val Tyr Glu Met  
 385 390 395 400

Gln Leu Met Ala Ser Lys Gly Tyr Tyr Val Val Phe Val Asn Pro Arg  
 405 410 415

Gly Ser Asp Gly Tyr Ser Glu Asp Phe Ala Leu Arg Val Leu Glu Arg  
 420 425 430

Thr Gly Leu Glu Asp Phe Glu Asp Ile Met Asn Gly Ile Glu Glu Phe  
 435 440 445

Phe Lys Leu Glu Pro Gln Ala Asp Arg Glu Arg Val Gly Ile Thr Gly  
 450 455 460

Ile Ser Tyr Gly Gly Phe Met Thr Asn Trp Ala Leu Thr Gln Ser Asp  
 465 470 475 480

Leu Phe Lys Ala Gly Ile Ser Glu Asn Gly Ile Ser Tyr Trp Leu Thr  
 485 490 495

Ser Tyr Ala Phe Ser Asp Ile Gly Leu Trp Tyr Asp Val Glu Val Ile  
 500 505 510

Gly Pro Asn Pro Leu Glu Asn Glu Asn Phe Arg Lys Leu Ser Pro Leu  
 515 520 525

Phe Tyr Ala Gln Asn Val Lys Ala Pro Ile Leu Leu Ile His Ser Leu  
 530 535 540

Glu Asp Tyr Arg Cys Pro Leu Asp Gln Ser Leu Met Phe Tyr Asn Val  
 545 550 555 560

Leu Lys Asp Met Gly Lys Glu Ala Tyr Ile Ala Ile Phe Lys Arg Gly  
 565 570 575

Ala His Gly His Ser Val Arg Gly Ser Pro Arg His Arg Pro Lys Arg  
 580 585 590

Tyr Arg Leu Phe Ile Glu Phe Phe Glu Arg Lys Leu Lys Lys Tyr Glu  
 595 600 605

Glu Gly Phe Glu Val Glu Lys Ile Leu Lys Gly Asn Gly Asn  
 610 615 620

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 50 NUCLEOTIDES
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR
- (ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCGAGAATTC ATTAAGAGG AGAAATTAAC TATGACCGGC ATCGAATGGA

50

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 33 NUCLEOTIDES
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR
- (ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AATAAGGATC CACTGGCA CAGTGCAAG ACA

33

What Is Claimed Is:

1. An isolated polynucleotide which encodes the amino acid sequence set forth in SEQ ID NO:2.
2. An isolated polynucleotide selected from the group consisting of:
  - a) SEQ ID NO:1;
  - b) SEQ ID NO:1, wherein T can also be U;
  - c) nucleic acid sequences complementary to a) and b); and
  - d) fragments of a), b), or c) that are at least 15 bases in length and that will hybridize to DNA which encodes the amino acid sequence of SEQ ID NO:2.
3. The polynucleotide of claim 1, wherein the polynucleotide is isolated from a prokaryote.
4. An expression vector including the polynucleotide of claim 1.
5. The vector of claim 4, wherein the vector is a plasmid.
6. The vector of claim 4, wherein the vector is a virus-derived.
7. A host cell transformed with the vector of claim 4.

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8. The host cell of claim 7, wherein the cell is prokaryotic.
9. The polynucleotide of claim 1 which encodes the enzyme comprising amino acid 1 to 622 of SEQ ID NO:2.
10. The polynucleotide of claim 1 comprising the sequence as set forth in SEQ ID NO:1 from nucleotide 1 to nucleotide 1866.
11. A substantially pure polypeptide selected from the group consisting of:
  - a) an enzyme comprising an amino acid sequence which is at least 70% identical to the amino acid sequence set forth in SEQ ID NO:2;
  - b) an enzyme which comprises at least 30 amino acid residues to the enzyme of a); and
  - c) the amino acid sequence as set forth in SEQ ID NO:2.
12. Antibodies that bind to the polypeptide of claim 11.
13. The antibodies of claim 12, wherein the antibodies are polyclonal.
14. The antibodies of claim 12, wherein the antibodies are monoclonal.



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15. A method for producing an enzyme comprising growing a host cell of claim 7 under conditions which allow the expression of the nucleic acid and isolating the enzyme encoded by the nucleic acid.
16. A process for producing a recombinant cell comprising transforming or transfecting the cell with the vector of claim 4 such that the cell expresses a polypeptide encoded by the DNA contained in the vector.
17. A process for removal of arginine phenylalanine or methionine from the N-terminal end of peptides in peptide or peptidomimetic synthesis, comprising: administering an amount of the enzyme of claim 10 effective for removal of arginine phenylalanine or methionine from the N-terminal end of peptides in peptide or peptidomimetic synthesis.

## Figure 1

## Thermococcus GU5L5 Amidase

1 ATG ACC GGC ATC GAA TGG AAC CAC GAG ACC TTT TCT AAG TTC GCC TAC  
 CTG GGC GAC CCG 60  
 1 Met Thr Gly Ile Glu Trp Asn His Glu Thr Phe Ser Lys Phe Ala Tyr  
 Leu Gly Asp Pro 20

61 AGG ATA CGG GGA AAC TTA ATC GCG TAC ACC CTG ACG AAG GCC AAC ATG  
 AAG GAC AAC AAG 120  
 21 Arg Ile Arg Gly Asn Leu Ile Ala Tyr Thr Leu Thr Lys Ala Asn Met  
 Lys Asp Asn Lys 40

121 TAC GAG AGC ACG GTT GTT GTT GAA GAC CTT GAA ACG GGC TCA AGG CGC  
 TTC ATC GAG AAC 180  
 41 Tyr Glu Ser Thr Val Val Val Glu Asp Leu Glu Thr Gly Ser Arg Arg  
 Phe Ile Glu Asn 60

181 GCC TCA ATG CCG AGG ATT TCG CCA GAC GGC AGA AAG CTC GCC TTC ACC  
 TGC TTT AAC GAG 240  
 61 Ala Ser Met Pro Arg Ile Ser Pro Asp Gly Arg Lys Leu Ala Phe Thr  
 Cys Phe Asn Glu 80

241 GAG AAG AAG GAG ACC GAG ATA TGG GTG GCC GAT ATC CAG ACC CTG AGC  
 GCC AAG AAA GTC 300  
 81 Glu Lys Lys Glu Thr Glu Ile Trp Val Ala Asp Ile Gln Thr Leu Ser  
 Ala Lys Lys Val 100

301 CTC TCA ACT AAA AAC GTC CGC TCG ATG CAG TGG AAC GAC GAT TCA AGG  
 AGA CTC TTA GTT 360  
 101 Leu Ser Thr Lys Asn Val Arg Ser Met Gln Trp Asn Asp Asp Ser Arg  
 Arg Leu Leu Val 120

361 GTC GGC TTC AAG AGG AGG GAC GAT GAG GAC TTC GTC TTT GAC GAC GAC  
GTC CCG GTC TGG 420

121 Val Gly Phe Lys Arg Arg Asp Asp Glu Asp Phe Val Phe Asp Asp Asp  
Val Pro Val Trp 140

421 TTC GAC AAT ATG GGA TTC TTT GAT GGA GAG AAG ACG ACG TTC TGG GTT  
CTT GAC ACT GAG 480

141 Phe Asp Asn Met Gly Phe Phe Asp Gly Glu Lys Thr Thr Phe Trp Val  
Leu Asp Thr Glu 160

481 GCC GAG GAG ATA ATC GAG CAG TTC GAG AAG CCG AGG TTT TCG AGT GGC  
CTC TGG CAC GGC 540

161 Ala Glu Glu Ile Ile Glu Gln Phe Glu Lys Pro Arg Phe Ser Ser Gly  
Leu Trp His Gly 180

541 GAT GCG ATA GTT GTG AAC GTC CCG CAC CGC GAG GGG AGC AAG CCT GCC  
CTG TTC AAG TTC 600

181 Asp Ala Ile Val Val Asn Val Pro His Arg Glu Gly Ser Lys Pro Ala  
Leu Phe Lys Phe 200

601 TAC GAC ATA GTC CTA TGG AAG GAC GGG GAG GAA GAG AAG CTC TTC GAG  
AGG GTC TCC TTC 660

201 Tyr Asp Ile Val Leu Trp Lys Asp Gly Glu Glu Glu Lys Leu Phe Glu  
Arg Val Ser Phe 220

661 GAG GCG GTT GAC TCC GAC GGA AAG AGA ATA CTC CTG AGG GGC AAG AAA  
AAA AAG CGG TTC 720

221 Glu Ala Val Asp Ser Asp Gly Lys Arg Ile Leu Leu Arg Gly Lys Lys  
Lys Lys Arg Phe 240

721 ATC AGC GAG CAC GAC TGG CTG TAC CTC TGG GAC GGC GAG CTT AAA CCG  
ATC TAC GAG GGC 780

241 Ile Ser Glu His Asp Trp Leu Tyr Leu Trp Asp Gly Glu Leu Lys Pro  
Ile Tyr Glu Gly 260

781 CCG CTC GAC GTC TGG GAA GCC AAG CTC ACG GAA GGA AAG GTC TAC TTC  
CTC ACT CCA GAT 840

261 Pro Leu Asp Val Trp Glu Ala Lys Leu Thr Glu Gly Lys Val Tyr Phe  
Leu Thr Pro Asp 280

841 GCG GGC AGG GTA AAC CTC TGG CTC TGG GAC GGG AAG GCC GAG CGT GTT  
GTT ACC GGC GAC 900

281 Ala Gly Arg Val Asn Leu Trp Leu Trp Asp Gly Lys Ala Glu Arg Val  
Val Thr Gly Asp 300

901 CAC TGG ATT TAC GGG CTT GAC GTC AGC GAT GGC AAA GCA TTG CTC CTC  
ATC ATG ACC GCC 960

301 His Trp Ile Tyr Gly Leu Asp Val Ser Asp Gly Lys Ala Leu Leu Leu  
Ile Met Thr Ala 320

961 ACG AGG ATA GGC GAG CTC TAC CTC TAC GAC GGC GAG CTG AAA CAG GTC  
ACC GAA TAC AAC 1020

321 Thr Arg Ile Gly Glu Leu Tyr Leu Tyr Asp Gly Glu Leu Lys Gln Val  
Thr Glu Tyr Asn 340

1021 GGG CCG ATA TTC AGG AAG CTC AAG ACC TTC GAG CCG AGG CAC TTC CGC  
TTC AAG AGC AAA 1080

341 Gly Pro Ile Phe Arg Lys Leu Lys Thr Phe Glu Pro Arg His Phe Arg  
Phe Lys Ser Lys 360

1081 GAC CTC GAG ATA GAC GGC TGG TAC CTC AGG CCG GAG GTT AAA GAG GAG  
AAG GCC CCG GTG 1140

361 Asp Leu Glu Ile Asp Gly Trp Tyr Leu Arg Pro Glu Val Lys Glu Glu  
Lys Ala Pro Val 380

1141 ATA GTC TTC GTC CAC GGC GGG CCG AAG GGC ATG TAC GGA CAC CGC TTC  
GTC TAC GAG ATG 1200

381 Ile Val Phe Val His Gly Gly Pro Lys Gly Met Tyr Gly His Arg Phe  
Val Tyr Glu Met 400

1201 CAG CTG ATG GCG AGC AAG GGC TAC TAC GTC GTC TTC GTG AAC CCG CGC  
GGC AGC GAC GGC 1260

401 Gln Leu Met Ala Ser Lys Gly Tyr Tyr Val Val Phe Val Asn Pro Arg  
Gly Ser Asp Gly 420

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1261 TAT AGC GAA GAC TTC GCG CTC CGC GTC CTG GAG AGG ACT GGC TTG GAG  
GAC TTT GAG GAC 1320

421 Tyr Ser Glu Asp Phe Ala Leu Arg Val Leu Glu Arg Thr Gly Leu Glu  
Asp Phe Glu Asp 440

1321 ATA ATG AAC GGC ATC GAG GAG TTC TTC AAG CTC GAA CCG CAG GCC GAC  
AGG GAG CGC GTT 1380

441 Ile Met Asn Gly Ile Glu Glu Phe Phe Lys Leu Glu Pro Gln Ala Asp  
Arg Glu Arg Val 460

1381 GGA ATA ACG GGC ATA AGC TAC GGC GGC TTC ATG ACC AAC TGG GCC TTG  
ACT CAG AGC GAC 1440

461 Gly Ile Thr Gly Ile Ser Tyr Gly Gly Phe Met Thr Asn Trp Ala Leu  
Thr Gln Ser Asp 480

1441 CTC TTC AAG GCA GGA ATA AGC GAG AAC GGC ATA AGC TAC TGG CTC ACC  
AGC TAC GCC TTC 1500

481 Leu Phe Lys Ala Gly Ile Ser Glu Asn Gly Ile Ser Tyr Trp Leu Thr  
Ser Tyr Ala Phe 500

1501 TCG GAC ATA GGG CTC TGG TAC GAC GTC GAG GTC ATC GGG CCA AAT CCG  
TTA GAG AAC GAG 1560

501 Ser Asp Ile Gly Leu Trp Tyr Asp Val Glu Val Ile Gly Pro Asn Pro  
Leu Glu Asn Glu 520

1561 AAC TTC AGG AAG CTC AGC CCG CTG TTC TAC GCT CAG AAC GTG AAG GCG  
CCG ATA CTC CTA 1620

521 Asn Phe Arg Lys Leu Ser Pro Leu Phe Tyr Ala Gln Asn Val Lys Ala  
Pro Ile Leu Leu 540

1621 ATC CAC TCG CTT GAG GAC TAC CGC TGT CCG CTC GAC CAG AGC CTT ATG  
TTC TAC AAC GTG 1680

541 Ile His Ser Leu Glu Asp Tyr Arg Cys Pro Leu Asp Gln Ser Leu Met  
Phe Tyr Asn Val 560

1681 CTC AAG GAC ATG GGC AAG GAA GCC TAC ATA GCG ATA TTC AAG CGC GGC  
GCC CAC GGC CAC 1740

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561 Leu Lys Asp Met Gly Lys Glu Ala Tyr Ile Ala Ile Phe Lys Arg Gly  
Ala His Gly His 580

1741 AGC GTC CGC GGA AGC CCG AGG CAC AGG CCG AAG CGC TAC AGG CTC TTC  
ATA GAG TTC TTC 1800

581 Ser Val Arg Gly Ser Pro Arg His Arg Pro Lys Arg Tyr Arg Leu Phe  
Ile Glu Phe Phe 600

1801 GAG CGC AAG CTC AAG AAG TAC GAG GAG GGC TTT GAG GTA GAG AAG ATA  
CTC AAG GGG AAT 1860

601 Glu Arg Lys Leu Lys Lys Tyr Glu Glu Gly Phe Glu Val Glu Lys Ile  
Leu Lys Gly Asn 620

1861 GGG AAC TGA 1869

621 Gly Asn End 623

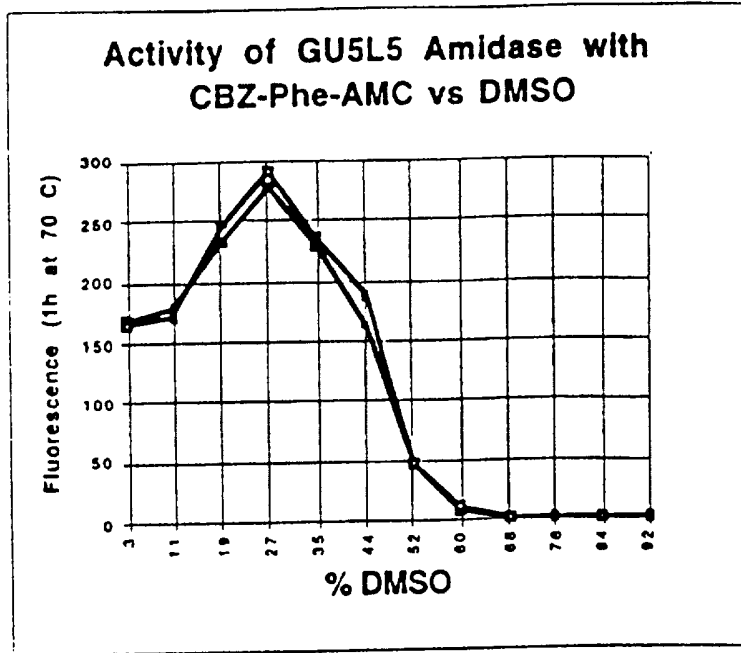


Figure 2

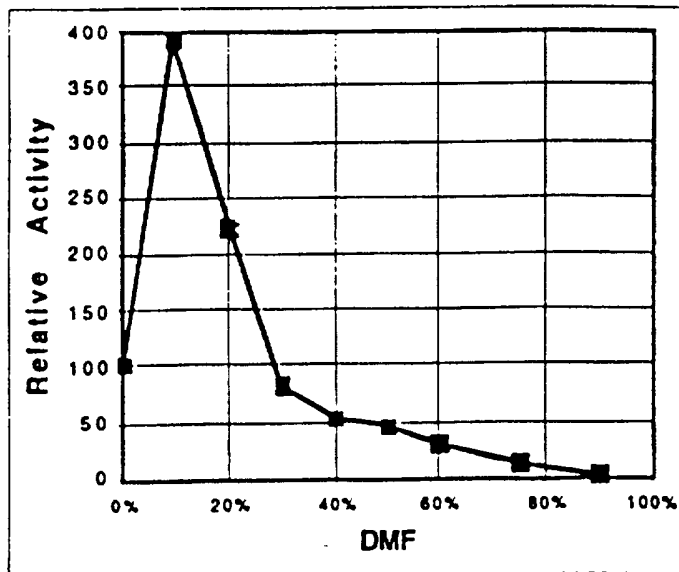


Figure 3

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/09319

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C12N 9/80, 15/00, 1/20; C12P 21/06; C07H 21/04; C07K 16/00  
US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/228, 69.1, 252.3, 320.1, 68.1; 536/23.2, 23.7; 530/387.1, 388.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
MParch pp - protein database search - geneseq25.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,451,522 A (QUEENER et al.) 19 September 1995, see entire document.	1-17

Further documents are listed in the continuation of Box C.

See patent family annex.

\* Special categories of cited documents:

\*A\* document defining the general state of the art which is not considered to be of particular relevance

\*E\* earlier document published on or after the international filing date

\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

\*G\* document member of the same patent family

Date of the actual completion of the international search

11 AUGUST 1997

Date of mailing of the international search report

03 SEP 1997

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

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Authorized officer

Tekchand Saidha

Telephone No. (703) 308-0196



**INTERNATIONAL SEARCH REPORT**

International application No.  
**PCT/US97/09319**

**A. CLASSIFICATION OF SUBJECT MATTER:**

US CL :

435/228, 69.1, 252.3, 320.1, 68.1; 536/23.2, 23.7; 530/387.1, 388.1

**B. FIELDS SEARCHED**

Electronic data bases consulted (Name of data base and where practicable terms used):

APS Search; STN : Files- Caplus, Medline, Wpids, Scisearch, Biosis, Biotechds.

Search Terms: Thermococcus, Amidase, gene or DNA or nucleic acid, antibod?, 7-amido-4-methylcoumarin? thermostable enzyme?, optically active isomer? and combinations thereof.