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(57) Abstract

A thermostable proteolytic enzyme which is producible by Thermoactinomyces thalipophilus THM1 (NCIMB 40778) and which:
(a) has a pH optimum of about 8; (b) has a temperature optimum of about 70 °C; (c) is substantially inhibited by 1 mM
phenylmethylsulphonylfluoride but substantially not inhibited by 10 mM dimethylsulphoxide, diethioleitol or β-mercaptoethanol; (d) is at
least partially dependent on the presence of calcium for its thermostability; and (e) is thermostable at 50 °C for at least 6 hours without
substantial loss of activity, is useful in the preparation of protein hydrolysates for nutritional and clinical use.
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THERMOSTABLE PROTEOLYTIC ENZYME FROM THERMOACTINOMYCES THALPOPHILUS THM1

This invention relates to micro-organisms capable of producing thermostable extracellular proteolytic enzymes. It also relates to the enzymes themselves, their preparation and use in the preparation of protein hydrolysates.

Proteins which have been partially digested by food-grade enzymes (i.e. protein hydrolysates) have been widely used as nutritional supplements for many years, for three main groups of patients.

The malabsorbing patient Prior hydrolysis converts the protein to a mixture of peptides and amino acids which may be better absorbed by patients with intestinal digestive disorders. It has been known for some years that the intestinal digestion products of protein are absorbed by the intestine in two forms, that is as free amino acids and as di- and tripeptides. There are two distinct transport mechanisms involved which are shown in the Figure 1.

The cells in the centre of the picture represent the absorptive cells lining the wall of the intestine. Di- and tripeptides are absorbed intact (Craft et al., Gut 9 425-437 (1968); Ferraris et al., Am. J. Physiol. 257 G689-G697 (1989); Nicholson et al., Clin. Sci. Mol. Med. 54 205-207 (1978); Nicholson et al., Eur. J. Clin. Invest. 9 349-354 (1979); Silk Gut 15 494-501 (1974); and Silk et al., Br. J. Nutr. 33 95-100 (1979)) and will be hydrolysed by cellular peptidases to release free amino acids which will be transported into the blood.

The allergic patient Predigestion of proteins also reduces the allergic potential of the protein, and is thus of benefit to infants with hypersensitivity to milk proteins. Since the allergic potential of a hydrolysate decreases as the size of its constituent peptides also decreases, this type of di- and tripeptide-based protein hydrolysate can be applied to these patients.

The post-operative patient By converting a large protein to smaller peptides, prior digestion markedly increases solubility. This is a useful property when trying to administer energy- and protein-dense liquid feeds to patients, with the added benefit that it allows acid flavourings (e.g. citrus which would otherwise curdle whole protein) to be added to these energy-dense food supplements, which has been shown to reduce the characteristic 'taste-fatigue' experienced by cancer patients towards milky supplements with sweet flavours.
There is another important application of protein hydrolysates, which is as a source of amino acids for intravenous nutrition (Total Parenteral Nutrition - TPN). Formulation shortcomings of some crystalline L-amino acid solutions (e.g. cysteine and tyrosine have poor solubility, whilst glutamine has poor stability), have led to considerable effort being put into soluble and stable synthetic dipeptides containing these problem amino acids. A simpler approach would be to use protein hydrolysates containing di- and tripeptides as a much cheaper alternative to synthetic peptides, and even to free L-amino acids.

Dipeptides have half the osmolarity of the same solution weight of free amino acids. They are therefore less irritating to veins if infused intravenously. At present, intravenous nutrition solutions consist of a mixture of amino acids and glucose in a fat emulsion, together with minerals and vitamins. These solutions are hypertonic and are too irritant to infuse into a peripheral vein. They are therefore infused through a catheter which is surgically implanted under the collar bone and into a large central vein, enabling the high blood flow to dilute the solution (Peripheral Parenteral Nutrition -- PPN). This technique has its risks and clinicians prefer infusing nutrient solutions into peripheral (e.g. arm) veins. This is a safer technique, even though it requires nutrient mixtures which are rather more dilute and therefore nutritionally inadequate. The use of complete mixtures of di- and tripeptides (from hydrolysates) would allow a more complete nutrient composition to be infused in this way.
In order to prepare protein hydrolysates, which as shown above are a clinically useful means of providing protein, it is in practice necessary to use an appropriate protease.

Proteases are one of the half-dozen major industrial and food enzymes. The proteases used for food use are fungal (e.g. Aspergillus proteases in condiment preparation and Mucor proteases in cheese-making), plant (e.g. papain), mammalian (e.g. pancreatic proteases) and to a lesser extent bacterial (e.g. Bacillus proteases). The constraints on use of any of these may be either legislative (i.e. a "safe" source is necessary) or functional (i.e. the stability, activity and specificity of the enzyme are important in generating the correct product).

Current manufacturing techniques for protein hydrolysates have shortcomings, particularly the use of slaughterhouse derived (i.e. pancreatic) or plant derived enzymes which tend to produce hydrolysates with a wide range of peptide chain-lengths. This is clearly demonstrated in the tabulated summary of hydrolysates which are currently used in liquid foods for 'malabsorbing' patients (Table 1).
Table 2. Peptide chain-length profiles of Protein Hydrolysates used in Enteral Diets

<table>
<thead>
<tr>
<th>Hydrolysate (Source)</th>
<th>Higher MW content</th>
<th>Di- &amp; Tri-peptide content</th>
<th>L-amino acids content</th>
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<tr>
<td>SURVIMED (Fresenius)</td>
<td>72</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>TRAVASORB (Clintech)</td>
<td>60</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>AMIRIGE (Nutricia)</td>
<td>59</td>
<td>26</td>
<td>15</td>
</tr>
<tr>
<td>PEPTI-2000 (Nutricia)</td>
<td>56</td>
<td>27</td>
<td>17</td>
</tr>
<tr>
<td>PEPTISORB (E. Merck)</td>
<td>44</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>STERALDIET (Dubenard)</td>
<td>46</td>
<td>29</td>
<td>25</td>
</tr>
<tr>
<td>REABILAN (Roussei)</td>
<td>65</td>
<td>33</td>
<td>2</td>
</tr>
<tr>
<td>TIEPTID (Lab. Roger Bellon)</td>
<td>23</td>
<td>69</td>
<td>8</td>
</tr>
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</table>

Peptide profiles measured by Cu(I) - SEPHADEX chromatography.
[Some of these diets may no longer be available or may be marketed under other names.]

Although the optimum absorptive properties for protein hydrolysates have been known for some time, only one hydrolysate in Table 1 (TIEPTID™) conforms to this. The technological issue is therefore not what the final product should be, but how this may be produced most economically.

As a class, proteases and peptidases exhibit a wide-range of activities, especially in their ability to cleave at specific amino acid residues in the native protein. This gives the possibility of predicting the peptide products of protein hydrolysis. Since the nature of the final end-product is known (di- and tripeptides), this can be achieved by careful choice of enzyme(s).

It has now been found that there exists a micro-organism which produces an enzyme which is particularly suitable
for producing protein hydrolysates for, among other things, clinical use.

According to a first aspect of the invention, there is provided a strain of *Thermoactinomyces thalpophilus* designated THM1.

*Thermoactinomyces thalpophilus* THM1 has been designated as strain HB 2682 in the Applicant's internal collection of strains and deposited under the terms of the Budapest Treaty at the National Collection of Industrial and Marine Bacteria Limited (NCIMB), Auris Business Centre, 23 St Macher Drive, Aberdeen AB2 1RY, on 18 December 1995 and given accession number NCIMB 40778. The deposit was found to be viable on 18 December 1995.

*T. thalpophilus* is a species within the genus *Thermoactinomyces*, the only genus within the group *Thermoactinomycetes*. *Thermoactinomyces* spp. are gram positive bacteria which are aerobic and saprophytic chemo-organotrophs. Most members of the genus are, like *T. thalpophilus*, thermophilic. Strains of *T. thalpophilus* have been isolated from soil, hay, cereal grain, sugar cane bagasse, cotton, grass compost, mushroom compost, peat, air conditioners and manure.

The invention encompasses *T. thalpophilus* THM1 and microorganisms derived from it. *T. thalpophilus* THM1 can be used in the derivation of other strains of microorganisms by any of the usual methods of microbiology, including mutation (prepared for example by ultra violet light or other radiation or by chemical mutagensis), environmental selection, classical genetic transfer or recombinant DNA techniques.
The micro-organisms of the invention produce a thermostable proteolytic enzyme whose properties are singularly useful in the preparation of protein hydrolysates.

According to a second aspect of the invention, there is provided a thermostable proteolytic enzyme which is producible by Thermoaactinomycetes thalpophilus THM1 (NCIMB 40778).

This heat-stable "thermophilic" protease has two major advantages to those of the current art:

(i) the heat stability of the enzyme allows hydrolysis reactions to be carried out at elevated temperatures, increasing reaction rates and reducing the danger of microbial contamination; and

(ii) it shows under defined conditions a marked tripeptide specificity (i.e. it will not hydrolyse proteins to amino acids), an important criterion for the preparation of nutritional hydrolysates.

In particular, the enzyme may be characterised in that it:

(a) has a pH optimum of about 8;
(b) has a temperature optimum of about 70°C;
(c) is substantially inhibited by 1mM phenylmethylsulphonylfluoride but substantially not inhibited by 10mM dimethylsulphoxide, dithiothreitol or β-mercaptoethanol;
(d) is at least partially dependent on the presence of calcium for its thermostability; and
(e) is thermostable at 50°C for at least 6 hours without substantial loss of activity.

Additionally, the enzyme has been found to have a pI of above 8.

Extracellular proteases from a number of thermophilic Actinomycetes strains have been identified, purified and partially characterised. The characterisation of an alkaline protease from an alkalophilic *Thermoactinomyces* isolate (HS682) has been reported (Tsuchiya et al., *Biosc. Biotech. Biochem.* 56(2), 246-250 (1992)). This enzyme shows properties broadly similar to the specific enzyme disclosed herein (inhibition by PMSF, stabilisation by calcium) but has a significantly higher pH optimum (11.5-13.0). Furthermore, the HS682 source organism, although not identified to species level, has very different physiological characteristics to the organism claimed herein, with a pH optimum for growth of 10.3 and a maximum growth temperature of 60°C (Tsuchiya et al., *Agric. Biol. Chem.* 55(12), 3125-3127 (1992)).

A protease purified and partially characterised from an isolate assigned the taxonomy of *Thermoactinomyces thalpophilus* (Odibo & Obi, *MIRCEN J.* 4 327-332 (1988)) showed characteristics significantly different from the enzyme specifically described herein. Whereas the specific enzyme has been identified as a calcium-stabilised serine protease, the prior reported T. *thalpophilus* enzyme is said to be a neutral protease inhibited by heavy metals but not by the covalent serine reagent diisopropylfluorophosphate.
A thermostable serine protease isolated from Thermoactinomyces vulgaris (Thermitase) has been studied in considerable detail (Leuchtenberger et al., Z. Allgem. Mikrobiol. 19(1) 27-35 (1979); Frömmel & Höhne, Biochim. Biophys. Acta 670 25-31 (1981); Kretschmer et al., Z. Allgem. Mikrobiol. 22 (10) 693-703 (1982); Behnke et al., Z. Allgem. Mikrobiol. 22 (8) 511-519 (1982); Brömme & Kleine Curr. Microbiol. 11, 93-100 (1984). However, Thermitase is substantially different from the enzyme specifically described herein, both on the basis of functional properties and because of the taxonomic difference of the source organism.

It can therefore be concluded that the enzymes the subject of this application (specifically, a thermostable serine protease derived from the thermophilic Actinomycete Thermoactinomyces thalpophilus), are substantially and significantly different from proteases previously reported.

The invention therefore provides in a third aspect a serine protease derivable from T. thalpophilus.

An enzyme of the invention may be produced by culturing a micro-organism as described above under conditions which cause expression (and preferably secretion) of the enzyme; suitable conditions include the use of a growth medium including glutamate. The enzyme may be harvested from the culture medium.

Enzymes of the invention may be substantially pure preparations, as determined for example by iso-electric focusing, SDS-PAGE, sequencing or crystallisation, for example. Alternatively, they may be impure preparations
or crude extracts, such as ammonium sulphate precipitations or partially purified fractions (eg gel filtration or chromatography fractions. The purity of the enzyme preparation will ultimately depend on its intended use.

Also included within the scope of the invention are preparations of enzyme which are modified in some way as compared to the natural enzyme. For example, the enzyme may be covalently or non-covalently immobilised onto an appropriate immobilisation matrix. Alternatively or additionally, the enzyme may be modified in its amino acid sequence (either chemically or by site-directed mutagenesis); but enzymes within the scope of the invention will generally be substantially (ie at least 60, 70, 80, 90, 95 or even 99%, in increasing order of preference) homologous to the prototype enzyme. Fusion proteins including an enzyme of the invention are also contemplated.

As mentioned above, the enzymes of the invention are useful in the preparation of protein hydrolysates.

According to a fourth aspect of the invention, there is provided a method of preparing a protein hydrolysate, the method comprising digesting one or more proteins with an enzyme as described above.

The digestion may be carried out substantially to completion, in which case the product will contain a high proportion of low molecular weight peptides (particularly di- and tri-peptides) or it may be terminated before completion, in which case it will contain a lower proportion of low molecular weight peptides and a higher
proportion of high molecular weight peptides. Longer hydrolysis products are preferred for the production of pharmaceutical and/or nutritional products.

In any event, the product will, in relation to the starting material(s) be enriched in di- and tripeptides and virtually free of single amino acids.

A wide variety of proteins and protein mixtures are suitable for being digested by the enzymes of the invention. For the preparation of protein hydrolysates for nutritional use, milk proteins may be used, particularly lactalbumin, such as bovine lactalbumin. More or less pure bovine lactalbumin preparations may be used; other proteins useful as substrates include other milk proteins (such as caseins) and plant proteins (such as soy proteins). Substrate proteins may be pure or impure preparations (containing, for example, lipidaceous material) and may be in a native or denatured state.

There are few critical parameters of the hydrolysis reaction. The reaction volume may be any volume which is convenient or desirable. The reaction time depends on the substrate, the temperature, other reaction conditions and the desired end point but will typically be from 30 minutes to 48 hours. The reaction temperature will typically be from 20°C to 100°C and is preferably from 45°C to 80°C. The enzyme concentration may be from 0.1 to 1000 units/ml, preferably from 5 to 50 units/ml; and the substrate concentration may be from 0.1% to 80% (w/v), preferably from 1 to 20%.

The invention also relates to a protein hydrolysate preparable by the above method, as well as to food and/or
pharmaceutical preparations comprising such a hydrolysate. Other envisaged uses include cosmetic and diagnostic uses and the use of protein hydrolysates as agents for modifying texture or surface coating. Fields of use are within the molecular biological, medical and pharmacological research, medical diagnostics and therapy, and the pharmaceutical, biotechnological, chemical and food industries.

For example, protein hydrolysates are incorporated in many food, dietary and cosmetic preparations for the purposes of enhancing physical, organoleptic and nutritional qualities. In analytical and research processes, protein hydrolysates may be used to prepare for use microtitre plates or blotting membranes. Protein hydrolysates have potential application in medical diagnostics as targets for drug screening or in receptor ligand binding. In the chemical industries, protein hydrolysates may be used for multiple purposes including the modification of the physical properties of emulsions such as paints, incorporation in polymeric substances and coatings for modern materials.

Preferred features of each aspect of the invention are as for each other aspect, mutatis mutandis.

The following description relates to a particularly preferred embodiment of the invention.

A micro-organism was isolated from a dry soil sample collected from a dry bed of a thermal stream at the Taupo thermal area in New Zealand. The micro-organism grows optimally at 50-55°C and shows proteolytic activity in casein agar plates. The micro-organism was identified as
a strain of *Thermoactinomyces thalophilus* by morphological and physiological characterisation. The novel strain is induced to produce extracellular protease by growth in a nutrient medium containing Na-glutamate. The extracellular proteolytic activity is enriched by fractionated ammonium-sulphate precipitation. Most of the activity is found in the 50-80% (NH₄)₂SO₄ fraction. This fraction gives after dialysis and ultrafiltration an enzyme preparation of about 3000u/ml as assayed with azocasein as substrate. The enzyme is most active at a pH of 8.0 and at a temperature of 70°C. The enzyme belongs to the family of serine proteases as shown by its 98.4% inhibition by phenylmethylsulphonylfluoride (PMSF) at 1 mM.

Ethylenediaminotetraacetic acid (EDTA) does not inhibit the enzymatic activity of the novel proteolytic enzyme but destabilises the enzyme, thereby reducing its thermal stability. It may be that calcium ions are necessary to stabilise the folding of the protein for its activity at high temperatures and possibly protecting against autolysis.

The enzyme finds particular application in the hydrolysis of the milk proteins, at alkaline pH, for the production of hydrolysates enriched in di- and tripeptides.

The invention will now be illustrated by the following examples. The examples refer to the accompanying drawings, in which:

FIGURE 1, which has already been referred to, schematically illustrates pathways of amino acid absorption in the human intestine;
FIGURE 2 refers to Example 10.2 and shows the effect of pH on THM1 protease;

FIGURE 3 refers to Example 10.3 and shows the temperature optimum of THM1 protease;

FIGURE 4 refers to Example 10.6 and shows the effect of calcium on THM1 protease; and

FIGURE 5 refers to Example 10.7 and shows the effect of temperature on the thermostability of THM1 protease.

Example 1: Isolation of the micro-organism

A dry soil sample from a dry bed of a thermal stream at the Taupo thermal area in New Zealand was resuspended in sterile distilled water. Approximately 50μl of the suspended sample was spread on a Nutrient Agar plate and incubated at 55°C overnight. After incubation a large white colony was picked from this plate and screened for protease production.

Example 2: Screen for extracellular proteolytic activity

The isolated micro-organism from Example 1 was subsequently tested for extracellular protease activity by culturing on a casein agar plate consisting of casein 2%, tryptone 0.5%, yeast extract 0.3%, agar 15%, pH 7.2, at 55°C overnight. The isolated organism produces extracellular protease activity as shown by zones of casein denaturation and progressive clearing by proteolytic activity.
Example 3: Characterisation of the micro-organism
A total of six related cultures from The National Collection of Industrial and Marine Bacteria (NCIMB) were obtained for comparison with Thermoactinomyces thalophilus THM1 strain. All cultured organisms were grown on CYC agar containing Czapek Dox liquid medium 33.4g/l, yeast extract 2g/l and Casamino acids 6g/l. The gross morphology and the presence of two extracellular growth metabolites, pigment and proteolytic activity, were examined. Proteolytic activity was detected on Casein agar (CA) as previously described. Cultures from NCIMB were T. thalophilus 9780, 11365, 11367, 11368, 11370, T. vulgaris 11364. See Table 2 below.

Table 2: Colony morphology of THM1 and NCIMB cultures

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<tr>
<th>Strain</th>
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<td>+</td>
<td>circular</td>
<td>umbonate</td>
</tr>
<tr>
<td>11368</td>
<td>-</td>
<td>+</td>
<td>circular</td>
<td>umbonate</td>
</tr>
<tr>
<td>11370</td>
<td>-</td>
<td>+</td>
<td>circular</td>
<td>umbonate</td>
</tr>
<tr>
<td>11364</td>
<td>-</td>
<td>(-)</td>
<td>filamentous</td>
<td>flat</td>
</tr>
</tbody>
</table>

THM1 grows with white aerial mycelium at 50°C on CYC agar. On this agar the organism shows a slightly dark pigmented substrate mycelium and produces a white aerial mycelium in 24 hrs. THM1 is able to grow on CYC agar containing tyrosine 5 mg/ml and novobiocin 25 μg/ml. Melanin was produced after 2-3 days. The ability to grow on CYC agar containing novobiocin at 50°C is characteristic of Thermoactinomycetes species and the
production of melanin on CYC agar containing tyrosine is characteristic of *Thermoactinomyces thalpophilus* and *Thermoactinomyces putidus*.

THM1 was able to grow on CYC agar in the presence of 200 mM NaCl. On agar containing Nutrient broth No. 2 powder (Oxoid, UK) 12.5 g/l, agar powder 20 g/l (¼ strength nutrient agar) and Arbutin 1 g/l or ¼ Strength nutrient agar containing Esculin 1 g/l and 0.05% Ferric Ammonium Citrate no coloration is produced indicating that THM1 is Arbutin and Esculin minus. These latter characteristics and the resistance to NaCl indicate that THM1 is a strain of *Thermoactinomyces thalpophilus* (T. Cross, 'The monosporic Actinomycetes' pp 2091-2102 in 'The Prokaryotes').

**Example 4: THM1 Protease is Different from Thermitase at the Genomic Level**

Two oligonucleotide primers were designed by reverse translation of conserved regions of serine proteases, particularly thermitase (Meloun et al., *FEBS Lett.* 183 195-200 (1985)) and the *Thermoactinomyces* species protease published by Lee et al., *Biosci. Biotechnol. Biochem.* 60 840-6 (1996), using the codon usage bias of *Thermoactinomycetes*. The first region chosen spans amino acid positions 14 to 21 in the thermitase sequence (SWISS-PROT P04072) and 154 to 161 in the *Thermoactinomyces* protease sequence published by Lee et al. (EMBL U31759), respectively. The second region covers the serine protease active site, amino acid positions 225 to 232 in the thermitase sequence and 367 to 374 in the *Thermoactinomyces* protease sequence published by Lee et al., respectively. Primers having the sequences 5'-GGA CCG CAA AAA GTC CAA GCT CCC-3' (SEQ
ID NO:1) and 5'-CCT GCC ACA TGC GGT GTG GCC AT-3' (SEQ ID NO:2) were synthesised. The two oligonucleotides were used for PCR amplification of a DNA fragment (predicted length of 662 bp), using genomic DNA from the different Thalphilus strains obtained from the National Collection of Industrial and Marine Bacteria (NCIMB) and THM1 as template. As a positive control DNA from T. vulgaris, also obtained from the NCIMB collection (NCIMB 11364) was used. The genomic DNAs were isolated according to standard procedures (Sambrook et al. Molecular Cloning, 2nd ed., Cold Spring Harbor Laboratory Press (1989)). PCR amplification of the DNA samples yielded a band of the expected size only with T. vulgaris DNA as a template. The negative result indicates that the THM1 protease is different from thermitase at the genomic level.

**Example 5: Preparation for Deposit at NCIMB**

Strain THM1 was grown in 50mL of CYC medium overnight at 50°C. The culture was concentrated 10-fold by centrifugation (5 minutes at 1000 rpm) and resuspended in CYC medium. 500µL of the suspension was added to a sterile lyophilisation tube and dried overnight under vacuum. The lyophilisation tube was sealed under vacuum and forwarded, at room temperature, to NCIMB, by whom it was received on 18 December 1995 and allocated deposit number NCIMB 40778.

**Example 6: Isolation of proteolytic activity**

The partial enrichment of the proteolytic enzyme was performed using ammonium sulphate precipitation. A fresh culture of the microorganism was grown on a Nutrient Agar plate overnight at 50°C. A single colony was picked and inoculated into 50 mL of sterile Nutrient broth. The
culture was incubated with shaking at 300rpm overnight at 50°C. To 1000 mL of protease enrichment broth containing sodium glutamate 1.5%, KH₂PO₄ 0.045%, tryptone 0.2%, yeast extract 0.05%, and CaCl₂ 0.147%, pH 7.0, 1 mL of the overnight culture was added and the culture incubated for 24 hours at 50°C with shaking at 200rpm. After incubation the culture was first sampled and 100μl of cell free broth checked for protease activity using the azocasein protease assay at 50°C, (see Example 6). The positive culture was centrifuged at 8000xg for 30 min to remove cells. The supernatant was retained, transferred to a 2L beaker and slowly stirred at 4°C. Under stirring (NH₄)₂SO₄ was added slowly to give 50% saturation and left for 3 hours. The broth was centrifuged at 8000xg for 30 min and both the pellet and supernatant retained. To the supernatant, (NH₄)₂SO₄ was added to 80% saturation and left overnight at 4°C with slow stirring. The liquid was centrifuged as before and the pellet retained. The second pellet was resuspended in 15 mL 0.1 M Tris buffer, 10mM CaCl₂, pH 8.0. For desalting and concentration the resuspended pellet was placed in an Amicon Centricon™ 10 ultrafiltration unit and centrifuged at 2500xg for 45 min. The protein solution was desalted 4 times by repeated addition of buffer to 15mL and centrifugation as above with a subsequent concentration of the solution down to a total of 3mL. The proteolytic activity of the protein isolate was assayed as described in Example 10.1 using 100 μl of a 1/200 dilution of the preparation.

The total activity contained in this preparation was found to be 8985U compared to 20500 U for the culture broth, giving a yield of 43.8%.
Example 7: Yield Improvement of THM Protease in Stimulating Broth

The improvement of yield of THM1 protease was studied in broth containing ingredients reported to stimulate the expression of proteolytic enzymes. The optimized broth (PPB) has the following composition per litre medium:

- Na glutamate: 15.0 g
- Tryptone: 2.0 g
- Yeast extract: 0.5 g
- KH$_2$PO$_4$: 0.45 g

Growth conditions in this medium were optimized in 2 L flasks on a shaking platform. The optimal conditions were found as 50 °C, pH 7.0, and 48 hrs, without excessive aeration (about 125 rpm). Optimal yields achieved after 2 days were about 3000 U/L.

Example 8: Preparative isolation of THM1 protease

A 15 L fermentation of Thermoactinomyces thalpophilus THM1 was performed in a fermenter of nominal volume of 16 L over 46 hours and the extracellular protease activity determined as described in Example 6.1. The fermentation conditions were as follows: 15 L PPB medium, t = 50°C, air 7.5 Lpm, pH controlled at 7.5, stirring rate 200 rpm. The cells were harvested by filtration and the culture broth filtered over a 10 kDa ultrafiltration membrane (Sartorius EASY FLOW$^\text{TM}$). The filtrate was concentrated using a 3 kDa ultrafiltration membrane (Amicon YM3) in a TCF10 unit at a N$_2$ pressure of 40 psi. The yields of protease activity at each step are shown in Table 3.
TABLE 3 - Yields of Protease Activity During isolation of THM1

<table>
<thead>
<tr>
<th>Experimental Step</th>
<th>Activity (U/ml)</th>
<th>Total Activity (Units)</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>fermentation broth</td>
<td>151.2</td>
<td>2268000</td>
<td>100</td>
</tr>
<tr>
<td>cleared broth</td>
<td>147.6</td>
<td>1845000</td>
<td>81.4</td>
</tr>
<tr>
<td>10 kDa ultrafiltrate</td>
<td>145.2</td>
<td>1815000</td>
<td>80.0</td>
</tr>
<tr>
<td>3 kDa ultrafiltration</td>
<td>11460</td>
<td>1719000</td>
<td>75.8</td>
</tr>
</tbody>
</table>

Example 9: Purification by Hydrophobic Interaction Chromatography

The protease was purified on Phenyl-Sepharose using a double gradient elution system.

A sample of 2 ml of THM1 protease (6000 U/ml, specific activity 5.5 U/µg) was applied at a flow rate of 2 ml/min to a 20 ml Phenyl-Sepharose column, pre-equilibrated with Tris·Cl 50mM, CaCl₂ 20mM, pH 8.0 (buffer A). The column was washed with 5 volumes of buffer A, and a gradient of 1-100% of buffer B (Tris·Cl 50mM, CaCl₂ 20mM, NaCl 1M, pH 8.0) over 5 column volumes. The enzyme was eluted with a second gradient using buffer B and buffer C (Tris·Cl 50mM, CaCl₂ 20mM, NaCl 1M, acetone 20%). The eluate was fractionated and the fractions tested for activity as described in Example 6.1
Out of 12000 units applied to the column, a total of 7500 units were recovered. The final yield was 62.5% of initial activity loaded. The specific activity of the activity recovered was measured as 12 U/μg protein, a 2.2-fold purification.

**Example 10: Characterisation of the proteolytic enzyme.**
The parameters examined of the enzyme isolated were pH and temperature optimums, thermostability, protease type by inhibition and isoelectric point.

**10.1 Azocasein protease assay**
All assays performed to determine proteolytic activity used azocasein as the substrate. Azocasein 0.2%, in an appropriate buffer and respective pH, was added to Eppendorf tubes in aliquots of 900μl and preincubated at the desired temperature for about 30 seconds before initiation of the reaction. A volume of 10-100μl of sample containing the proteolytic enzyme was added to the substrate and the reaction left to proceed for 10 minutes. Then 500μl of trichloroacetic acid 15% was added to terminate the reaction. The tubes were left on the bench to cool for a further 10 minutes, to allow precipitation of unhydrolysed azocasein, then centrifuged for 2 minutes. The supernatant was removed and placed into plastic 1ml cuvettes and the absorbance read at 420nm. One unit of proteolytic activity is defined as ΔOD₄₂₀·h⁻¹·ml⁻¹ of 1.00.

**10.2 pH Optimum**
The pH optimum of the enzyme preparation was examined using azocasein 0.2% as substrate dissolved in a multi-pH buffering solution consisting of 50mM Na₂HPO₄, 50mM H₃BO₄,
50mM CH₃COOH, adjusted to the pH values of 5, 6, 7, 8, 9, 10 and 11 with 5M NaOH. Aliquots of 900μl of the buffered azocasein were preincubated at 70°C in duplicate microcentrifuge tubes. A 100μl aliquot of 1/200 diluted concentrated THM1 enzyme preparation was added to initiate each reaction. The assay then proceeded as previously described. The results are shown in Figure 2.

10.3 Temperature Optimum
900μl aliquots of azocasein 0.2%, tris 100 mM, pH 8.0 were placed in duplicate into microcentrifuge tubes and preincubated at different temperatures from 30 to 100°C. A 100μl aliquot of 1/200 diluted of protease preparation were added and the assay continued as described above. The results are shown in Figure 3.

10.4 Inhibition of THM1 Protease
The inhibition of the partially purified protease was examined using the serine protease inhibitor, phenylmethylsulphonylfluoride (PMSF) and the thiol compounds, dimethylsulphoxide (DMSO), dithiolthreitol (DTT) and β-mercaptoethanol. A 10μl aliquot of 1/10 dilution of the THM1 enzyme preparation was incubated with the desired concentration of inhibitory reagent in tris buffer 0.1M pH 8.0 to a total of 100μl at room temperature for 30 minutes. After incubation the azocasein assay was performed, as described before, using the 100μl reaction volume. The change in absorbance at 420nm was determined and compared to an enzyme only control. See Table 4.
Table 4. Effect of Inhibitors on THM1 protease.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Residual Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSF (1mM)</td>
<td>1.6</td>
</tr>
<tr>
<td>PMSF (2mM)</td>
<td>1.6</td>
</tr>
<tr>
<td>PMSF (5mM)</td>
<td>1.5</td>
</tr>
<tr>
<td>PMSF (10mM)</td>
<td>1.5</td>
</tr>
<tr>
<td>DMSO (10mM)</td>
<td>91.4</td>
</tr>
<tr>
<td>DTT (10mM)</td>
<td>73.1</td>
</tr>
<tr>
<td>β-mercaptoethanol (10mM)</td>
<td>84.9</td>
</tr>
</tbody>
</table>

10.5 Molecular Mass of Protease THM1

The molecular mass of the protease of THM1 was analysed on a 15% SDS-PAG. A sample containing 5μg of purified THM1 protease (Example D) was incubated either in the presence of 40 mM PMSF or 10 mM EDTA for 30 min. at room temperature. SDS-sample buffer was added and the samples boiled for 2 minutes. The samples were loaded onto the gel and electrophoresed at 25mA for 2.5 hours. Staining with Coomassie Brilliant Blue showed a single band in the sample incubated in the presence of PMSF (lane 1) and in addition a smaller degradation product in the sample incubated in the presence of EDTA. The major band shows a molecular mass of about 32 kDa as determined from comparison with marker proteins.
10.6 Effect of calcium on the thermostability of THM1 protease:

In a final volume of 200 μl, CaCl_2 10 mM or EDTA 20 mM, 20 μl of THM1 protease preparation and an appropriate volume of tris 100 mM pH 8.0 were added to microcentrifuge tubes. The initial activity of each was assayed using 100 μl of reaction mixture and azocasein 0.2% for substrate, as previously described, then the remaining volume was incubated at temperatures from 40 to 100 °C for 30 minutes and the final activity assayed as before. The residual activity of the enzyme was determined by comparing initial and final activities and is shown in Figure 4.

10.7 Effect of temperature on the thermostability of THM1 protease:

A 10 fold dilution of THM1 protease preparation was prepared in Tris 100 mM pH 8.0 to final volumes of 500 μl which were incubated at 50, 60, 70 and 80 °C. Aliquots of 10 μl were taken at the beginning of the incubation and then hourly for 6 hours. The samples were assayed using the azocasein 0.2% protease assay at 70 °C method as stated in Example 10.1. The initial activity of each was determined to be 100% and the residual activity calculated. Results are shown in Figure 5.

10.8 pI:

The isoelectric point of the enzyme was determined using the Pharmacia PhastSystem™ Electrophoresis unit. Two PhastGel isoelectrophoresis gels, pH 3-9, were set up according to the Pharmacia PhastSystem manual and loaded
identically with 1μl of protease preparation and electrophoresed according to the IEF program. After electrophoresis, one gel was stained with coomassie blue using the Pharmacia protocol and the second gel was checked for activity by incubating with 10 ml Casein 2%, Tris 100mM pH 8.0 for 30 minutes at 50°C. The resultant activity detected on the gel incubated with casein substrate aligned with a band detected on the coomassie blue stained gel which was located in comparison to standards at a pI of above 8.

10.9: Size Estimation by Gel Filtration

A SUPEROSE 12 HR 10/30 size exclusion column was equilibrated with buffer A (50 mM Tris·Cl, pH 7.5, 100 mM KCl). The column was calibrated with dextran blue (Sigma D4772, 1 mg/ml) and protein standards (cytochrome C, Sigma C7150, 2 mg/ml; carbonic anhydrase, Sigma C7025, 2 mg/ml; apoprotinin, Sigma A3886, 3 mg/ml; albumin, Sigma A8531, 5 mg/ml) in 200 μl buffer B (50 mM Tris·Cl, 100 mM KCl, 5% v/v glycerol) at 0.1 ml/min.

200 μl THM-1 protease (1.025 mg/ml; 3.4 U/μg) in buffer B were applied to the column and eluted with buffer A at 0.1 ml/min. Twenty 0.25 ml fractions were collected and activity determined as described. No activity was detectable in fractions 1 to 12; the total applied activity was recovered in fractions 13 - 17, indicating an apparent molecular mass of 8 kDa as determined from the elution profile of the marker proteins. The active fractions were pooled, freeze dried and the protease resuspended in 250 μl Tris·Cl, pH 6.8, yielding a protein
concentration of 430 \( \mu g/ml \). To further determine the molecular mass of the protease a discontinuous SDS-PAGE according to Schägger and von Jagow (Anal. Biochem. 166 368-379 (1987)) was performed with the obtained pooled fraction. 15 \( \mu l \) were taken up in 15 \( \mu l \) sample buffer, denatured by boiling for 2 min., and electrophoresed on a 16.5% acrylamide gel at 30 mA constant current. After staining in Coomassie Blue a single band was visible which migrated at about 32 kDa in comparison with marker proteins (Sigma MW-SDS-17S and Promega V5231). This result is in agreement with earlier size estimations as shown in Example 10.5.

Example 11: Proteolytic digestion of Bovine Lactalbumin by THM1 Protease.

Method 1

A 5% suspension of insoluble, denatured bovine lactalbumin substrate was prepared in \( K_2HPO_4 \) 0.1M, pH 10 CaCl\(_2\) 5mM with penicillin-G 20\( \mu g/ml \) to suppress bacterial contamination. A 20ml volume of substrate was incubated with 20units/ml of THM1 protease at 50°C with gentle shaking in a 50ml conical flask for a total of 24 hours. Aliquots of 1ml were taken at various time points. The pH of the reaction mixture was continually checked and readjusted to pH 10 using 1M NaOH if required.

Method 2

The digestion reaction was performed identically as stated above except that the pH was modified to pH 9.5.
The products of the reaction were analysed as described in Example 12. The results shown in Table 5.

**Example 12:** Analysis of Protein substrate digestions via Cu\(^{II}\)-Sephadex column chromatography and total acid hydrolysis/OPA-MESNA assay.

### 12.1 Cu\(^{II}\)-SEPHADEX \(^{\large \text{TM}}\) chromatography

A Cu\(^{II}\)-SEPHADEX \(^{\large \text{TM}}\) column was prepared as follows. 80 mL of copper sulphate 0.08 M was added to 21.3 g SEPHADEX fine G-25, and left overnight at room temperature. 40 mL NaOH 1M was added with vigorous stirring to initiate the formation of a copper complex with the SEPHADEX. The suspension was centrifuged, the supernatant and any copper hydroxide layer removed and a further 40 mL of NaOH 1M was added. The SEPHADEX complex was recentrifuged then washed twice with Na\(_2\)B\(_4\)O\(_7\) buffer 50 mM, pH 11.5 and centrifuged. The pellet was then resuspended with about 20-50 mL of the borate buffer and two columns were poured, a 40 cm column and a 5 cm precolumn. The columns were put in place at 4°C and equilibrated using the said borate buffer, degassed with 100% helium, with a 1 mL/min flow rate monitored at a 280 nm wavelength. Proteolytic digestion samples of 0.5 mL were loaded and fractions were collected for further analysis. A corresponding molecular size profile was plotted from detection of elution products to which three molecular size categories were assigned. See Table 5.
12.2 Total acid hydrolysis of chromatographic fractions.

Fractions collected from Cu²⁺-SEPHADEX chromatography were first neutralized to pH 7.0 with 5M HCl then passed through a mini-column containing 5 mL CHELEX-100® resin (Sigma Chemical Co., Poole) equilibrated in borate buffer 50 mM, pH 11.5 to remove bound copper. The column was then eluted with 2 mL of the equilibration buffer. A volume of 2 mL of the collected eluant was lyophilized overnight. The resulting powder was redissolved in 1ml of water. 0.5 ml was taken as the before hydrolysis sample. 0.5 ml of 12M HCl was added to the remaining sample and incubated overnight at 110°C. 1 mL of NaOH, 5M was added to neutralize any remaining acid and the final volume brought to 2ml. Both the before and after total acid hydrolysis samples were co-assayed for amino nitrogen using the OPA-MESNA assay method detailed in Example 12.3.

12.3 OPA-MESNA assay of chromatographic fractions.

Stocks of o-phthaldialdehyde (OPA) 40mM (10x concentration) in methanol and 2-mercaptoethanesulphonic acid (MESNA) in Na₂B₄O₇ buffer 100mM pH 10.5 (1x concentration) were prepared. An appropriate volume of sample from either the before or after total acid hydrolysis samples was added and adjusted to a final volume of 400μl with borate buffer 50mM pH 10.5. Then 200μl of MESNA was added followed by 200μl of 1x OPA reagent. The reaction was incubated at room temperature for 5 minutes and then an absorbance at a wavelength of 334nm. To determine sample concentration, the reading was compared to a standard. The ratio of amino nitrogen
concentrations before and after total acid hydrolysis resulted in the average chain length of peptides present with each column fraction collected. See results in Table 6 for proteolytic digests of lactalbumin Methods 1 and 2.

Table 5. Molecular weight profile of Lactalbumin digestion by THM1 Protease

<table>
<thead>
<tr>
<th>Method</th>
<th>High Molecular weight peptides %</th>
<th>Low molecular weight peptides %</th>
<th>Amino Acids %</th>
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<tr>
<td>1</td>
<td>60</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>40</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 6. Average Peptide Chain Lengths in fractions of Lactalbumin digestion by THM1 protease

<table>
<thead>
<tr>
<th>Fraction Number</th>
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<th>Method 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average Peptide Chain Length</td>
<td>Average Peptide Chain Length</td>
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</tr>
<tr>
<td></td>
<td>10</td>
<td>2.3</td>
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</table>

Example 13: Comparative Digestion by Proteolytically Active Broth Extracts of Different Thermoactinomyces thalpophilus Strains

Protease containing extracts of culture broths of Thermoactinomyces thalpophilus THM1 and the NCIMB bacterial strains of T. thalpophilus 9780, 11365, 11367, 11368 and 11370 were prepared using ammonium sulphate precipitation and ultrafiltration concentration. The activity of the concentrates was assayed using the azocasein method. A comparative digestion was performed using bovine lactalbumin (Sigma) and the broth extracts at an activity of 40 U/ml.
A stock of 6.25% insoluble lactalbumin substrate (Sigma) was prepared in glycine buffer 0.1 M, CaCl\textsubscript{2} 5 mM pH 10.0. To 100 ml conical flasks, 20 ml of the substrate, penicillin-G 20 μg/ml, the appropriate volume of extract and glycine buffer to a total of 25ml were added and the pH adjusted to pH 10. The flasks were incubated for 11 hrs on a shaking platform at 50°C and 100 rpm. The pH was controlled and adjusted to pH 10, initially every 30 min., than every hour.

1 ml samples were taken to determine the digestion profile on Cu\textsuperscript{II}-SEPHADEX G25 column as described.

**Table 7**

Comparative digestion by *T. thalpophilus* proteases

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Time (hours)</th>
<th>HMW (%)</th>
<th>Di/tripeptide (%)</th>
<th>Amino acids (%)</th>
<th>Substrate digested (%)</th>
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</thead>
<tbody>
<tr>
<td>THM1</td>
<td>11</td>
<td>82</td>
<td>18</td>
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<td>9780</td>
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<td>61</td>
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<td>11370</td>
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<td>12</td>
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<td>-ve control</td>
<td>11</td>
<td>92</td>
<td>8</td>
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<td>1</td>
</tr>
</tbody>
</table>

(Notes: HMW = high molecular weight; percentage values for HMW, di/tripeptides and amino acids are relative and do not include total substrate usage)
SEQUENCE LISTING

(1) GENERAL INFORMATION:

5  (i) APPLICANT:
(A) NAME: Helix Biotechnology Ltd
(B) STREET: 182 Gloucester Place
(C) CITY: London
(D) STATE: -
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): NW1 6DS

(A) NAME: Anderson, Jasen Kingsley
(B) STREET: c/o Helix Biotechnology Ltd,
Darwin Building, Gower Street
(C) CITY: London
(D) STATE: -
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): WC1E 6BT

20  (A) NAME: Grimble, George Kenneth
(B) STREET: c/o Helix Biotechnology Ltd,
Darwin Building, Gower Street
(C) CITY: London
(D) STATE: -
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): WC1E 6BT

(A) NAME: Cowan, Donald Arthur
(B) STREET: c/o Helix Biotechnology Ltd,
Darwin Building, Gower Street
(C) CITY: London
(D) STATE: -
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): WC1E 6BT
(ii) TITLE OF INVENTION: THERMOSTABLE PROTEOLYTIC ENZYME FROM Thermoactinomyces thalpophilus THM1

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
   (A) MEDIUM TYPE: Floppy disk
   (B) COMPUTER: IBM PC compatible
   (C) OPERATING SYSTEM: PC-DOS/MS-DOS
   (D) SOFTWARE: WordPerfect 5.1 for DOS

(v) CURRENT APPLICATION DATA:
   APPLICATION NUMBER: WO PCT/GB 96/_____

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 24 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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

GGACCGCAAA AAGTCCAAGC TCCC

1       11       21
INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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

CCTGCCACAT GCGGTGTGGC CAT
1 11 21
INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 6, line 8-15.

B. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet

Name of depositary institution

THE NATIONAL COLLECTIONS OF INDUSTRIAL AND MARINE BACTERIA LIMITED

Address of depositary institution (including postal code and country)

23 St. Machar Drive, Aberdeen AB2 1RY, Scotland, UK

Date of deposit
16 December 1995

Accession Number
NCIMB 40778

C. ADDITIONAL INDICATIONS (leave blank if not applicable)

This information is continued on an additional sheet

Where permitted by law, and in particular under EPC Rule 28(4), the microorganism referred to above may be made available only by the issue of a sample to an expert.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

For receiving Office use only

This sheet was received with the international application

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For International Bureau use only

This sheet was received by the International Bureau on:

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Form PCT/RO/134 (July 1992)
CLAIMS

1. *Thermoactinomyces thalpophilus* THM1 (NCIMB 40778).

2. A micro-organism which is derived from *Th. thalpophilus* THM1 (NCIMB 40778).

3. A thermostable proteolytic enzyme which is producible by *Thermoactinomyces thalpophilus* THM1 (NCIMB 40778).

4. A thermostable proteolytic enzyme producible by a micro-organism of the genus *Thermoactinomyces* which:

   (a) has a pH optimum of about 8;

   (b) has a temperature optimum of about 70°C;

   (c) is substantially inhibited by 1mM phenylmethysulphonylfluoride but substantially not inhibited by 10mM dimethylsulphoxide, dithiothreitol or β-mercaptoethanol;

   (d) is at least partially dependent on the presence of calcium for its thermostability; and

   (e) is thermostable at 50°C for at least 6 hours without substantial loss of activity.

5. A serine protease derivable from *T. thalpophilus*. 
6. A process for the preparation of a thermostable proteolytic enzyme, the process culturing a microorganism as claimed in claim 1 or 2 under conditions which cause expression of the enzyme.

7. A process as claimed in claim 6, wherein the microorganism is cultured in the presence of glutamate.

8. A process as claimed in claim 6 or 7, wherein the enzyme is harvested from the culture medium.

9. A method of preparing a protein hydrolysate, the method comprising digesting one or more proteins with an enzyme as claimed in claim 3, 4 or 5.

10. A method as claimed in claim 9, wherein one or more milk proteins are digested.

11. A method as claimed in claim 10, wherein the milk protein is lactalbumin.

12. A protein hydrolysate preparable by a method as claimed in claim 9, 10 or 11.

13. A food preparation comprising a hydrolysate as claimed in claim 12.
PATHWAYS OF AMINO ACID ABSORPTION IN THE HUMAN INTESTINE
FIGURE 2

Effect of pH on THM1 Protease

Relative Activity (%) vs pH
FIGURE 3

Temperature Optimum of THM1 Protease.

Relative Activity (%)

20 40 60 80 100

20 40 60 80 100 120

Temperature (°C)

FIGURE 4

Effect of Calcium on THM1 Protease

Residual Activity (%)

0 20 40 60 80 100

30 50 70 90 110

Temperature (°C)

- calcium 10 mM
- EDTA 20 mM
FIGURE 5

Effect of Temperature on the Thermostability of THM1 Protease

Residual Activity (%)

Time (hrs)

- 50 degrees celcius
- 60
- 70
- 80
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N/52 C12N/20 C12P21/06 A23J3/00 A61K38/01
//(C12N1/20,C12R1:01)

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)

IPC 6 C12N C12P A23J A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>X</td>
<td>BIOSCI BIOTECHNOLOG BIOCHEM, FEB 1992, 56 (2) P246-50, JAPAN, XP000670147</td>
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<td>TSUCHIYA K ET AL: &quot;Purification and characteriziation of a thermostable alkaline protease from alkophilic Thermotoga sp. HS6B2.&quot; cited in the application see page 248 - page 249</td>
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<td>DD 262 675 A (BERLIN CHEMIE VB) 7 December 1988 see example 1</td>
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<td>WO 96 28558 A (PROCTER &amp; GAMBLE) 19 September 1996 see the whole document</td>
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Further documents are listed in the continuation of box C. Patent family members are listed in annex.

Date of the actual completion of the international search 17 April 1997

Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2400, Tx. 31 651 epo nl, Fax (+31-70) 340-3016

Authorized officer Espen, J

Date of mailing of the international search report 25.04.97

Form PCT/ISA/210 (second sheet) (July 1992)

page 1 of 2
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<td>WO 9628558 A</td>
<td>19-09-96</td>
<td>AU 5182996 A</td>
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