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(54) Title: X-PROLYL-DIPEPTIDYL-AMINOPEPTIDASE FROM <i>LACTOBACILLUS DELBRÜCKII</i> SSP. <i>LACTIS</i> , NUCLEIC ACIDS CODING FOR THE SAME AND ITS USE IN FERMENTED FOODSTUFF PREPARATION PROCESSES (57) Abstract The invention relates to a protein as produced and excreted by <i>Lactobacillus delbrückii</i> ssp. <i>lactis</i> and capable of releasing X-Pro dipeptides from the N-terminus of oligopeptides present in the culture medium of said lactobacilli, or fragments thereof having said releasing X-Pro dipeptides activity. The invention relates also to the use of said protein or fragments thereof for the preparation of fermented foodstuff, and more particularly of cheese.		

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X-PROLYL-DIPEPTIDYL-AMINOPEPTIDASE FROM *LACTOBACILLUS DELBRÜCKII* SSP. *LACTIS*, NUCLEIC ACIDS CODING FOR THE SAME AND ITS USE IN FERMENTED FOODSTUFF PREPARATION PROCESSES

The invention relates to X-Prolyl-dipeptidyl-aminopeptidase (PepX) from *Lactobacillus delbrückii* ssp. *lactis* or polypeptides derived thereof, and more particularly recombinant PepX from said *Lactobacillus* strain or recombinant polypeptides derived thereof, which can be used in fermented foodstuff preparation processes.

The invention also relates to processes for preparing the PepX from said *Lactobacillus* strain or said derived polypeptides, which are in a state of biological purity such that they can be used in processes for the preparation of fermented foodstuff.

The invention also relates to nucleic acids coding for PepX from said *Lactobacillus* strain or said derived polypeptides, and to cellular hosts containing said nucleic acids and their use in processes for the preparation of fermented foodstuff.

Furthermore, the invention relates to processes for the preparation of fermented foodstuff and kits using the PepX from said *Lactobacillus* strain or said derived polypeptides, and/or said cellular hosts.

By "recombinant polypeptides" it is to be understood that it relates to any molecule having a polypeptidic chain liable to be produced by genetic engineering, through transcription and translation of a corresponding DNA sequence under the control of appropriate regulation elements within a efficient cellular host. Consequently the expression "recombinant polypeptides" such as is used herein does not exclude the possibility for the polypeptides to comprise other groups, such as glycosylated groups.

The term "recombinant" indeed involves the fact that the polypeptide has been produced by genetic engineering, particularly because it results from the expression in a cellular host of the corresponding nucleic acid sequences which have previously been introduced into the expression vector used in said host.

Nevertheless, it must be understood that this expression does not exclude the possibility for the polypeptide to be produced by a different process, for instance by classical chemical synthesis according to methods used in the protein synthesis or by proteolytic cleavage of larger molecules.

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The expression "biologically pure" or "biological purity" or "in a substantially pure form" means on the one hand a grade of purity such that the polypeptide or recombinant polypeptide can be used for the preparation of fermented foodstuff and on the other hand the absence of contaminants, more particularly of natural contaminants.

Rapid growth of lactobacilli in milk depends of their ability to utilize milk proteins as an external source for essential amino acids. As the concentrations of small peptides and free amino acids in milk are rather low, lactobacilli dispose of various proteolytic and peptidolytic activities which are involved in the degradation of casein (1, 17, 31).

The PepX is capable of releasing X-Pro dipeptides from the N-terminus of oligopeptides. The structure of the amino acid proline influences the conformation of peptides and restricts the cleavage of proline-containing peptides by common exo- and endopeptidases. As casein has an extraordinarily high content of proline residues (16,7 % for β -casein) (8), the enzyme is supposed to be indispensable for efficient casein utilization.

Consequently the presence of the PepX has been demonstrated in a wide range of lactic acid bacteria (8). The enzyme was purified from *Lactobacillus lactis*, *Streptococcus thermophilus* (22), *Lactobacillus delbrückii* ssp. *bulgaricus* (2, 5, 23), *Lactobacillus helveticus* (13), *Lactobacillus acidophilus* (5), *Streptococcus mitis* (12), *Lactococcus lactis* ssp. *cremoris* (6, 14, 34), and *Lactococcus lactis* ssp. *lactis* (19, 36). The *pepX* gene of *Lactococcus lactis* ssp. *lactis* (24) and of *Lactococcus lactis* ssp. *cremoris* (21) was isolated and sequenced; these genes were found to be more than 99 % homologous.

An aspect of the invention is to provide a new family of nucleic acids coding for new proteins and polypeptides which can be used for the preparation of fermented foodstuff.

Another aspect of the invention is to provide cellular hosts transformed with said nucleic acids which can be used for the preparation of fermented foodstuff, and more particularly as starter organisms in the fermentation of milk.

Another aspect of the invention is to provide nucleic acids coding for the peptidic chain of biologically pure recombinant polypeptides which enable their preparation on a large scale.

Another aspect of the invention is to provide new proteins and polypeptides which can be used for the preparation of fermented foodstuff.

Another aspect of the invention is to provide processes for the preparation of fermented foodstuff, and more particularly cheese.

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The invention relates to a protein in a substantially pure form, as produced and excreted by *Lactobacillus delbrückii* ssp. *lactis* and capable of releasing proline-containing dipeptides (or X-Pro dipeptides) from the N-terminus of oligopeptides present in the culture medium of said lactobacilli, or fragments thereof having said releasing X-Pro dipeptides activity.

The invention relates more particularly to the protein (PepX) as defined above, such as produced by the strain of *Lactobacillus delbrückii* ssp. *lactis* WS87, deposited at the Deutsche Sammlung von Mikroorganismen (DSM) under the number 7290 on October 15, 1992, the polypeptidic sequence of said PepX being represented on figure 5 or figure 6. The amino acid sequence of PepX is also represented by SEQ ID NO 2 in the sequence listing which follows.

Said PepX represented by SEQ ID NO 2, is more particularly characterized in that:

- its isoelectric point calculated from the nucleotide sequence represented by SEQ ID NO 1, is 4.96, and its isoelectric point determined by preparative isoelectric focusing after ammonium sulfate fractionation of crude extract of said protein, is 3.9,

- its molecular weight calculated from the nucleotide sequence represented by SEQ ID NO 1, is 88.449 kDa, and its molecular weights determined by SDS-PAGE after purification, by SDS-PAGE after in vitro translation of plasmid pJK433 (see below), and by gel filtration, are 95 kDa, 89.9 kDa and 84 kDa respectively,

- its hydropathy profile is represented on figure 8,

- specific chromogenic substrates of said PepX are chosen among L-Ala-L-Pro-p-nitroanilide and Gly-L-Pro- β -naphthylamide,

- specific inhibitors of said PepX are the following:

- phenylmethylsulfonylfluoride (PMSF), end concentration 1 mM: 73 % relative activity,

- 3,4-dichloroisocoumarine (3,4-DCI): end concentration 0,1 mM: 55 % relative activity,

- 4-(2-aminoethyl)-phenylsulfonylfluoride, hydrochloride (AEBSF), end concentration 0,1 mM: 92 % relative activity.

The percentages of relative activity indicated above, correspond to residual activities after addition of the inhibitor as compared with the control without inhibitor. The conditions and methods for determination are taken from "Proteolytic enzymes, a practical approach, Beynon R.J. and Bond J.S., Oxford

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University Press 1989." (Chapter 4, Determination of protease mechanism, Ben M. Dunn).

The PepX according to the invention is more particularly characterized in that it is obtained from a cell extract of said *Lactobacillus delbrückii* ssp. *lactis* WS87 by the following four-step procedure:

- fractionation of the cell extract by salting out at 4°C with solid ammonium sulfate from 40 to 60 % saturation,

- hydrophobic interaction chromatography by applying the ammonium sulfate fraction obtained at the previous step to an appropriate column such as a column of Octyl-Sepharose 4B (Pharmacia), and pooling the eluted fractions having the highest specific activity against L-Ala-L-Pro-p-nitroanilide,

- ion exchange chromatography, by applying the pooled fractions obtained at the previous step to an appropriate column such as a Mono Q HR (Pharmacia),

- affinity chromatography using L-Ala-L-Pro linked to EAH-Sepharose 4B (Pharmacia) as ligand.

The degree of purity of the enzyme obtained by applying the process above-described is approximately of 98%.

The invention also relates to polypeptides derived from the PepX from said *Lactobacillus* strain defined above, said derived polypeptides corresponding to fragments of the polypeptide sequence represented by SEQ ID NO 2, or muteins (which differ from said protein by addition and/or substitution and/or suppression of one or several amino acid) thereof, provided that said fragments and muteins are capable of releasing X-Pro dipeptides.

Proteins or polypeptides derived thereof according to the invention are in the form of a monomer or a polymer, more particularly a dimer or a trimer.

Preferred proteins or polypeptides derived thereof according to the invention are in the form of a monomer.

The invention also relates to nucleic acids coding for proteins or polypeptides such as defined above.

The invention relates more particularly to the nucleic acid sequence represented by SEQ ID NO 1 coding for PepX, the amino acid sequence of this latter protein being represented under the nucleotide sequence coding for PepX in SEQ ID NO 1.

Nucleic acids according to the invention are more particularly characterized in that:

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- they comprise all or part of the nucleotidic sequence represented on figure 5 or figure 6, and corresponding to SEQ ID NO 1, coding for the PepX from said *Lactobacillus* strain, or its complementary sequence,

- or they hybridize with all or part of said nucleic acid represented by SEQ ID NO 1, or with its complementary sequence.

By way of illustration, the hybridization above-mentioned can be performed with membranes like HeybondTM-N (Amersham) or Hybridization Transfer Membrane (Micron Separations Inc.) under conditions as described by the supplier.

The invention also relates to recombinant nucleic acid containing at least one of the nucleic acids according to the invention, inserted in a heterologous nucleic acid.

The invention relates more particularly to recombinant vectors comprising a vector sequence, notably of the type plasmid (such as plasmids originating from lactic acid bacteria), cosmid or phage, and a nucleic acid according to the invention, in one of the non-essential sites for their replication.

Preferred recombinant vectors according to the invention, are those containing in one of their non essential sites for their replication, necessary elements to promote the expression of polypeptides according to the invention in a cellular host and possibly a promoter recognized by the polymerase of the cellular host, particularly an inducible promoter and possibly a signal sequence and/or an anchoring sequence.

Recombinant vectors can contain the elements enabling the expression by *E. coli* or lactic acid bacteria including *lactococcus* and, especially, lactobacilli of the thermophilic groups, of a nucleic acid according to the invention inserted in the vectors, to demonstrate product formation.

Particularly preferred recombinant vectors are constructed from replicons isolated from lactobacilli, especially from thermophilic *Lactobacillus* species.

The invention also relates to cellular hosts which are transformed by a recombinant vector such as defined above, and comprising the regulation elements enabling the expression of the nucleotide sequence coding for a protein according to the invention in these hosts.

Preferred cellular hosts are chosen from among bacteria such as *E. coli*, or from among lactic acid bacteria including *lactococcus* and, especially, lactobacilli of the thermophilic group, transformed by a vector according to the invention.

From the nucleic acids of the invention, probes (i.e. cloned or synthetic oligonucleotides) can be inferred.

These probes can be from 25 nucleotides up to the total length of the pepX gene. These oligonucleotides can also be used as amplification primers in the PCR technique (PCR, Mullis and Faloona, Methods in Enzymology, vol. 155, p. 335, 1987) to generate specific enzymatically amplified fragments and/or as probes to detect fragments amplified between bracketing oligonucleotide primers.

The invention also relates to expression products of nucleic acids expressed by transformed cellular hosts such as described above.

The invention also relates to a process for preparing a protein such as defined above comprising the following steps:

- the culture in an appropriate medium of a cellular host as described above,
- the recovery of the polypeptide produced by the above said cellular host from the above said culture medium,
- the purification of the polypeptide produced, more particularly according to the method above described.

The polypeptides of the invention can be prepared according to the classical techniques in the field of peptide synthesis.

The synthesis can be carried out in homogeneous solution or in solid phase.

For instance, the synthesis technique in homogeneous solution which can be used is the one described by Houbenweyl in the book entitled "Methoden der organischen Chemie" (Method of organic chemistry) edited by E. Wünsch, vol. 15-I and II, THIEME, Stuttgart 1974.

The polypeptides of the invention can also be prepared in solid phase according to the methods described by Atherton and Sheppard in their book entitled "Solid phase peptide synthesis" (IRL Press, Oxford, New York, Tokyo, 1989).

The invention also relates to a process for preparing the nucleic acids according to the invention.

A suitable method for chemically preparing the single-stranded nucleic acids (containing at most 100 nucleotides of the invention) can be carried out according to the automatic β -cyanoethyl phosphoramidite method of DNA synthesis described in Bioorganic Chemistry 4; 274-325 (1986).

In the case of single-stranded DNA, the material which is obtained at the end of the DNA synthesis can be used as such.

A suitable method for chemically preparing the double-stranded nucleic acids (containing at most 100 bp of the invention) comprises the following steps:

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- DNA synthesis of one sense oligonucleotide using the automatic β -cyanoethyl phosphoramidite method above cited, and DNA synthesis of one antisense oligonucleotide using either the above-mentioned automatic β -cyanoethyl phosphoramidite method, or enzymatic transcription of the sense-strand using a specific primer hybridizing to the 3'-end of the sense strand,

- combining the sense and antisense oligonucleotide by hybridization in order to form a DNA duplex,

- cloning the DNA duplex obtained into a suitable plasmid vector and recovery of the DNA according to classical methods such as restriction enzyme digestion and agarose electrophoresis, or by PCR amplification according to the procedure outlined above.

A method for the chemical preparation of nucleic acids with lengths greater than 100 nucleotides - or base pairs, in the case of double-stranded nucleic acids - comprises the following steps:

- assembling the synthesized oligonucleotides, provided at their ends with different restriction sites, the sequences of which are compatible with the succession of amino acids in the natural peptide, according to the principle described by Urdea et al. in Proc. Nat. Acad. Sci. USA 80; 7461-7465 (1983),

- cloning the DNA thereby obtained into a suitable plasmid vector and recovery of the desired nucleic acid according to classical methods such as restriction enzyme digestion and agarose gel electrophoresis.

The invention also relates to antibodies themselves formed against the polypeptides according to the invention, more particularly by immunization of appropriate animals with said polypeptides and recovery of antibodies thus formed.

It goes without saying that this production is not limited to polyclonal antibodies.

It also relates to any monoclonal antibody produced by any hybridoma liable to be formed according to classical methods from splenic cells of an animal, particularly of a mouse or a rat, immunized against the purified polypeptide of the invention on the one hand, and of cells of a myeloma cell line on the other hand, and to be selected by its ability to produce the monoclonal antibodies recognizing the polypeptide which has been initially used for the immunization of the animals.

The invention also relates to any antibody of the invention labeled by an appropriate label of the enzymatic, fluorescent or radioactive type.

The invention relates more particularly to the use of the polypeptides described above for the determination of the exact localization of PepX in

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Lactobacillus delbrückii ssp. *lactis*, rendering possible experiments performed by immunoblotting after cell fractionation and by electron microscopy of immunogold labeled peptidases.

The invention also relates to fermentation processes comprising a step of treatment of material to be fermented with:

- an appropriate amount of at least one of the protein according to the invention, and/or

- an appropriate amount of at least one of the transformed cellular host such as described above, and/or

- an appropriate amount of lactobacilli, such as *Lactobacillus delbrückii* ssp. *lactis*.

The invention also relates to a process for the preparation of fermented foodstuff, and more particularly of cheese, which comprises a step of treatment of food material to be fermented, such as milk, with:

- an appropriate amount of at least one of the protein according to the invention, and/or

- an appropriate amount of at least one of the transformed cellular host such as described above, and/or

- an appropriate amount of lactobacilli, such as *Lactobacillus delbrückii* ssp. *lactis*.

The process for the preparation of fermented foodstuff described above can also comprise a step of treatment of food material to be fermented, such as milk, with other species and strains susceptible to be used as starter organisms in fermentation processes, and more particularly lactic acid bacteria susceptible to produce a PepX protein, such as *Lactococcus*, *Streptococcus* and *Lactobacillus*.

The fermentation processes according to the invention, are more particularly characterized in that they can be used as fermentation processes for the obtention of hard cheeses, such as Emmentaler type cheese (South Germany, Switzerland).

The invention also relates to foodstuff, and more particularly cheeses, such as obtained by fermentation processes as described above.

Other characteristics and advantages of the invention will appear in the following examples and the figures illustrating the invention.

I) Figure legends

Figure 1:

Figure 1 represents the purification steps visualized on SDS-Gel. Lane 1: marker proteins; a myosin (205 kDa), b β -galactosidase (116 kDa),

c phosphorylase B (97.5 kDa), d bovine serum albumin (66 kDa), e ovalbumin (45 kDa); lane 2: crude extract of *Lactobacillus delbrückii* ssp. *lactis* WS87 (DSM 7290); lane 3: ammonium sulfate fractionation; lane 4; HIC-fraction; lane 5: Mono Q-fraction; lane 6: L-Ala-L-Pro-EAH-Sepharose-fraction.

5 Figure 2:

Figure 2a represents the effect of temperature on the activity of PepX.

The x-axis corresponds to the temperature (°C), and the y-axis corresponds to the relative activity (%).

Figure 2b represents the effect of pH on the activity of PepX. The x-axis corresponds to the pH, and the y-axis corresponds to the relative activity (%).

10 Figure 3:

Figure 3 represents the localization of *pepX* on the plasmid pJK433, and subcloned fragments in pUC18.

15 Figure 4:

Figure 4 represents the overexpression of PepX in *E. coli*. Lane 1: crude extract of JM109; lane 2: crude extract of JM109 (pLG339); lane 3: crude extract of JM109 (pJK433); lane 4: marker proteins, a myosin (205 kDa), b β -galactosidase (116 kDa), c phosphorylase B (97.5 kDa), d bovine serum albumin (66 kDa), e ovalbumin (45 kDa), f carbonic anhydrase (29 kDa).

20 Figure 5:

Figure 5 represents the nucleotide sequence (corresponding to SEQ ID NO 1) and deduced amino acid sequence (in the one-letter code) of PepX. the restriction sites used for subcloning and complementation experiments are indicated. Putative ribosome binding sites (RBS) and potential promotor regions are underlined. The convergent arrows above DNA sequence indicate the putative transcription terminator. Asterisk, stop codon. N-terminal amino acid sequence determined on the purified protein is overlined.

25 Figure 6:

Figure 6 represents the nucleotide sequence and deduced amino acid sequence (in the three-letter code) of PepX (corresponding to SEQ ID NO 2).

30 Figure 7:

Figure 7 represents a computer alignment of the amino acid sequences of the PepX from *Lactobacillus lactis* ssp. *lactis* WS87 (lbpepX), *Lactococcus lactis* ssp. *cremoris* (llxpdap, 21), and *Lactococcus lactis* ssp. *lactis* (llpepxp, 24). Identical amino acids (*) in the sequences are indicated.

Figure 8:

Figure 8 represents the hydropathy plot of PepX according to Kyte and Doolittle (15).

II) Materials and methods

1) Bacterial strains, plasmids, and media

Bacterial strains and plasmids are listed in Table 1. Lactobacillus delbrückii ssp. lactis was grown in MRS medium (20) at 42°C. *Escherichia coli* was grown in LB medium (3) at 37°C with shaking. When needed, kanamycin (40 µg/ml), ampicillin (200 µg/ml), or tetracycline (12.5 µg/ml) was added to the culture medium.

5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; Biomol, Hamburg) and isopropyl-β-thiogalactoside (IPTG; Biomol) were used at 0.004 % (wt/vol) and 0.2 mM, respectively.

2) Preparation of cell extract

Lb. delbrückii was grown at 42°C for 15 - 20 h in 10 l MRS broth. Cells were harvested by centrifugation at 11.000 g for 10 min at 4°C, and washed twice with 50 mM Tris hydrochloride buffer pH 7.2. By grinding with aluminium oxide at 4°C cells were disrupted and then suspended in 50 mM Tris hydrochloride buffer pH 7.2 (1.0 - 1.5 ml per g wet cells). Aluminium oxide was removed from the homogenate by centrifugation at 11,000 g and 4°C for 10 min. Absorbed proteins were extracted from aluminium oxide by washing with 50 mM Tris hydrochloride buffer pH 7.2 (0.5 - 0.75 ml per g wet cells), before the cell debris was down by ultracentrifugation at 139,000 g and 4°C for 60 min.

E. coli was grown overnight at 37°C with shaking in 100 ml LB medium.

Cells were harvested by centrifugation at 7,000 g for 10 min. The pellet was washed once with 20 ml 50 mM Tris hydrochloride buffer pH 7.5, suspended in 50 mM Tris hydrochloride buffer pH 7.5 with an end volume of the suspension of 1 ml. After disruption by ultrasonication (Bandelin electronic, type UW 60) cell debris were removed at 52,000 g and 4°C for 1 h.

3) Enzyme assays, protein assay

During purification PepX was routinely tested in microtiter plates with L-Ala-L-Pro-p-nitroanilide (Bachem, Bubendorf, Switzerland) as substrate. The reaction mixture contained 0.2 ml Tris hydrochloride pH 7.2. 10 µl substrate (10 mg/ml in water), and 10 µl enzyme solution. After incubation at 37°C the amount of p-nitroaniline liberated was determined at 405 nm. One unit of

enzymatic activity was defined as the amount of enzyme that liberates 1 μ mol p-nitroaniline per minute at 37°C. The specific activity was defined as units per milligramm of protein. Protein concentration was measured with the methods of Bradford (7) and Lowry (18), respectively.

During cloning of the gene, recombinant *E. coli* clones exhibiting PepX activity were screened with an enzymatic plate assay. The plates were flooded with fast garnet GBC (Sigma, St. Louis, Mo.) solution (1mg/ml in water) supplemented with Gly-L-Pro- β -naphthylamide (0.4 mg/ml; Bachem). The cleavage of the chromogenic substrate resulted in formation of an azodye which gives positive colonies a cherry-red appearance.

For determination of the protease type the following inhibitors were used: 3,4-dichloro-isocoumarine (3,4-DCI; Boehringer, Mannheim, Germany), phenylmethanesulphonylfluoride (PMSF; Sigma), pepstatin (novobiochem), 1,10-phenanthroline (Sigma), and L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64; Sigma).

4) Purification

- Step 1: *Ammoniumsulfate fractionation.*

The cell extract was fractionated by salting out at 4°C with solid ammonium sulfate from 40 to 60 % saturation. After gently mixing for 1 h at 4°C the precipitate formed was collected by centrifugation at 21,000 g and 4°C for 30 min and dissolved in a minimum amount of 50 mM Tris hydrochloride pH 7.2.

- Step 2: *Hydrophobic interaction chromatography (HIC)*

The undialysed ammonium sulfate fraction was applied at 4°C to a 26 by 1.5 cm column of Octyl-Sepharose 4B (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM Tris hydrochloride pH 7.2 in 1 M ammonium sulfate.

The enzyme was eluted (28.5 ml/h) with a linear salt gradient between equilibration buffer and distilled water. The fractions with the highest specific activity were pooled, dialysed against 20 mM Tris hydrochloride pH 7.2, and concentrated by passage through a PM30 Diaflo membrane (Amicon, Beverly).

- Step 3: *Ion exchange chromatography*

The pooled fractions of step 2 were applied to Mono Q HR 5/5 (Pharmacia) equilibrated with 20 mM Tris hydrochloride pH 7.2. Proteins were eluted at 1 ml/min with a two-step linear NaCl gradient: 0 to 0.25 M NaCl for 6 min, 0.25 to 0.5 M NaCl for 30 min.

- Step 4: *Affinity chromatography*

Carbodiimide coupling of L-Ala-L-Pro to EAH-Sepharose 4B (Pharmacia) was based on a procedure recommended by the supplier. After coupling of the

ligand a column was packed (Bio-Rad, Richmond, Calif.; gel bed 7 by 1 cm) and equilibrated at 4°C with 20 mM Tris hydrochloride buffer pH 7.2. The dialysed fraction of step 3 was applied. The column was washed with equilibration buffer and eluted (12 ml/h) with a linear salt gradient of 0 to 0.5 M NaCl.

5) Polyacrylamide gel electrophoresis

Crude extracts and enzyme preparations at each purification step were analysed by SDS-polyacrylamide gel electrophoresis using the system of Laemmli (16). Gels were stained with Coomassie blue according to Weber and Osborn (32).

Non-denaturing polyacrylamide gel electrophoresis (12 %) was carried out according to Davis (9). Activity staining was carried out by incubation of individual gels with 10 ml staining solution (1.5 % agar, 0.1 mM CoCl₂, 0.1 mM, MnCl₂, 0.5 mg/ml L-Ala-L-Pro-p-nitroanilide). The added agar prevented a too fast diffusion of the p-nitroaniline formed at the enzyme zone.

6) Gel filtration

The molecular weight of the native enzyme was estimated by gel filtration on Fractogel TSK HW-55(S) (Merck, Darmstadt, Germany ; 2.5 by 74 cm).

Elution with 50 mM Tris hydrochloride pH 7.5 was at 60 ml/h.

L-Alaninedehydrogenase (280 kDa), alkaline phosphatase (100 kDa), peroxidase (40 kDa), and myoglobine (17 kDa) were used as molecular markers.

7) Isoelectric focusing

Preparative isoelectric focusing after ammonium sulfate fractionation of crude extract was carried out in an apparatus of the firm LKB (Bromma, Sweden; type 8100-1) as described by the supplier. Ampholytes (Serva, Heidelberg, Germany) in the pH range of 3 to 6 were used. The pH gradient was stabilized by a sucrose gradient.

8) Sequencing of the N-terminus

For determination of the N-terminus preparative SDS-PAGE of a HIC-fraction was followed by electrotransfer (0.8 of mA/cm² for 2h) of PepX to a glass-fiber membrane (Glassybond; Biometra; Göttingen; Germany) as described by Eckerskorn (11). To prevent amino-terminal blocking of the protein, the time for polymerisation was prolonged and ammonium persulfate as well as N, N, N', N'-tetramethyl aminomethane were removed at 100 V for 1 h. N-terminal microsequencing in gasphase was performed by Appligene (Illkirch, France).

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9) Molecular cloning

Procedure for the isolation of chromosomal DNA, DNA manipulations, and cloning were essentially those as proposed by Sambrook-Maniatis-Fritsch (27). Isolation of plasmid DNA was performed according to Birnboim and Doly (4). Restriction endonucleases and T4 DNA ligase were obtained from Boehringer Mannheim, and as recommended by the supplier. Transformation of *E. coli* was done by electroporation with the Gene PulserTM (Bio-Rad Richmond, Calif.) as described by Dower et al. (10).

10) DNA sequence analysis

The determination of the nucleotide sequence was performed according to Sanger et al. (28) using double-stranded DNA template primed with specially synthesized oligonucleotides (392 DNA/RNA synthesizer, Applied Biosystems, Foster City, Calif.). The reported sequence was determined for both strands.

5'-[α -³⁵S]dATP was purchased from Amersham-Buchler (Buckinghamshire, United Kingdom), the T7 DNA polymerase sequencing kit was obtained from Pharmacia. DNA and amino acid sequence analysis were performed using the Microgenie sequence analysis software package (25).

11) Nucleotide sequence accession number

The GenBank and EMBL nucleotide sequence accession number is Z14230.

III) Results

1) Purification and characterisation

Table 2 summarizes the purification of PepX from *Lb. delbrückii* ssp. *lactis* WS87 to electrophoretic purity by use of a four-step procedure including ammonium sulfate fractionation, hydrophobic interaction chromatography on Octyl-Sepharose 4B, ion exchange chromatography on Mono Q HR 5/5, and affinity chromatography on L-Ala-L-Pro-EAH-Sepharose 4 B. Fig. 1 shows SDS-PAGE analysis of the enzyme fractions during purification. The final purification was approximately 200-fold, and recovery was 4.6 %. As estimated by SDS-PAGE and gelfiltration on HW TSK-55 (S), the enzyme is a monomer with a molecular mass of 95 kDa (SDS-PAGE) and 84 kDa (gel filtration). By isoelectric focusing after ammonium sulfate fractionation a pI of 3.9 was determined. Optimal PepX activity in crude extract was measured at pH of 7.0, and temperatures of 46-50°C (figures 2a and 2b respectively). Denaturation occurs at pH values below 4 and at temperatures above 55°C. The enzyme belongs to the class of serine proteases since it is inhibited by 3,4-dichloroisocoumarine and phenylmethanesulphonyl fluoride, specific

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inhibitors for serine proteases. The N-terminal amino acid sequence detected by microsequencing in gasphase is overlined in figure 5.

2) Cloning of the pepX gene

Due to the absence of the pepX gene in *E. coli* cloning was performed in this organism. Total DNA prepared from *Lb. delbrückii* ssp. *lactis* WS87 was partially digested with *Sau*III A endonuclease. DNA fragments of 4 - 10 kb obtained by size fractionation through a sucrose gradient (10 - 40 %) were ligated into the *Bam*HI restriction site of the *E. coli* low copy number vector pLG339. Using the substrate Gly-L-Pro- β -naphthylamide which could not be cleaved by the *E. coli* wildtype two recombinant *E. coli* ER1562 clones (2 out of 5000) exhibited respective PepX activity: pJK433 (6.3 kb insert) and pJK434 (6.7 kb insert). The restriction map of pJK433 which was further investigated is shown in figure 3.

3) Overexpression in *E. coli*

The PepX gene, designated pepX, was very efficiently expressed in the *E. coli* host strain from its authentic promotor. Overproduction could be visualized on SDS-PAGE : PepX constituted more than 50% of the cytoplasmatic proteins (figure 4). However, the plasmid pJK433 exhibited only limited stability in the heterologous *E. coli* system. Even in the presence of kanamycin, which selects for the presence of the plasmid, PepX activity was completely lost after approximately 100 generations.

4) Sequence analysis

The location of PepX on the 6.3 kb insert of pJK433 was determined by cleavage with appropriate restriction enzymes, religation, and subsequent complementation experiments (figure 3). Deletion of a 0.8 kb *Sph*I fragment, and of a 0.5 kb *Bam*HI-*Bgl*III fragment, respectively, resulted in loss of Gly-L-Pro-naphthylamide cleaving activity of the respective *E. coli* ER1562 transformants. Regions flanking the partial 4.8 kb *Sph*I fragment, on the other hand, were found to be dispensable to the functional integrity of the pepX gene.

We did not succeed in subcloning of the partial *Sph*I fragment into the high copy number vector pUC18, whereas the 0.8 kb *Sph*I fragment and the 4.0 kb *Sph*I fragment, respectively, could be ligated in to the *Sph*I restriction site of the pUC18 polylinker (figure 3). Constructing was performed and monitored in *E. coli* JM109 with X-Gal and IPTG. It seems that overexpression of PepX in a high copy number plasmid may be lethal to the host strain.

The nucleotide sequence of a 3,215 bp region was determined, and an open reading frame was found that starts at position 288 (AUG) and stops at the ochre termination codon (TAA) at position 2,664. Figure 5 shows the complete

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nucleotide sequence, the open reading frame detected encodes a protein of 792 amino acids with a calculated molecular mass of 88,449 Da. Upstream of the ORF, there are several alternative stretches of A's and T's, the AT-content is 65.5 % (position 1 - 287). It is less clear-cut, however, to assign the expression signals. A putative ribosome-binding site was found five bp upstream of the start codon, a potential -10 and -35 region with a spacing of 18 bp is underlined in figure 5. Downstream of the *pepX* gene a region of dyad symmetry located between the nucleotides 2,720 and 2,757 is capable of forming a stem loop of 17 bp (containing five GC pairs, $\Delta G = -33$ kcal/mol), and could act as a transcription terminator. The GC content of the DNA fragment sequenced is 53.1 % which agrees well with the GC value for *Lactobacillus delbrückii* (50 %) described by Weiss et al. (33).

The N-terminal amino acids of the protein specified by the ORF agree with those determined by amino acid sequencing of the purified enzyme. The hydrophilicity plot of the deduced amino acid sequence according to Kyte and Doolittle (15) does not show any appropriate segment at the N-terminus likely to be a signal peptide sequence, nor a transmembrane α -helical domain, or a membrane anchor.

Comparison of the *pepX* nucleotide sequence and of the deduced amino acid sequence with the EMBL databank shows significant homology with PepX from *Lactococcus lactis* (21, 24). Considering the gene, there was found 53 % identity over a stretch of 1,377 bp (position 516 to bp 1,919).

The gene product revealed 36 % identity over a stretch of 554 amino acids (position 28 to 682), according to the algorithm of Smith and Waterman (29) there was found 55 % similarity. Figure 6 shows a computer alignment of the three sequences. Apart from the lactococcal enzyme there was no significant homology detected with any protein of the EMBL data bank.

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Table 1: Bacterial strains and plasmids

5	Strain or plasmid	Relevant genotype or phenotype	Reference or source
	<u>STRAINS</u>		
10	<i>E. coli</i> JM109	<i>recA1, endA1, gyrA96, thi, hsdR17 supE44, relA1, λ⁻, Δ(lac-proAB), [F⁺, traD36, proAB, lacI^q ZΔM15]</i>	35 Pharmacia (Uppsala, Sweden)
15	<i>E. coli</i> ER1562	<i>λ⁻, endA1, thi-1, supE44, hsdR2, mcrB1, mcrA1272 : : Tn10, r⁻m⁺</i>	26 New England Biolabs GmbH (Schalbach, Germany,) # 401-H
20	<i>Lb. delbrückii</i> <i>ssp. lactis</i> WS87		Dr. M. Busse, Technische Universität München, Germany
25	<u>PLASMIDS</u>		
30	pLG339	Tc ^r , Km ^r , 6.2 kb, low copy number	30
	pUC18	Ap ^r , 2.68 kb, <i>lacZ</i> , pBR322 derivative, high copy number	35
35	pJK433	Km, 12.5 kb, partial <i>SauA</i> fragment low copy, <i>pepX</i>	this work

* Ap^r, Km^r, Tc^r, resistance to ampicillin, kanamycin and tetracycline, respectively.

Table 2: Purification of PepX from *lactobacillus delbrückii* ssp. *lactis* WS87

	Purification step	Activity [U/ml]	Specific activity [U/mg]	Total activity [U]	Recovery [%]	Purification [-fold]
5	Cell free extract	1.29	0.13	152.3	100	1
10	Ammonium sulfate	6.71	0.26	125.5	82.5	2
15	Octyl-Sepharose 4 B	37.3	3.55	44.8	29.4	27.3
20	Mono Q HR 5/5	1.82	15.17	14.6	9.6	116.7
25	Ala-Pro-EAH- Seph. 4 B	0.2	26	7.0	4.6	200

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SEQUENCE LISTING

5

(1) GENERAL INFORMATION:

(i) APPLICANT:

- 10 (A) NAME: COMMUNAUTE ECONOMIQUE EUROPEENNE
(B) STREET: Batiment Jean Monnet - Plateau du Kirchberg
(C) CITY: LUXEMBOURG
(E) COUNTRY: LUXEMBOURG
(F) POSTAL CODE (ZIP): L-2920

- 15 (ii) TITLE OF INVENTION: X-PROLYL-DIPEPTIDYL-AMINOPEPTIDASE FROM
LACTOBACILLUS DELBRUCKII SSP. LACTIS, NUCLEIC ACIDS CODING
FOR THE SAME AND ITS USE IN FERMENTED FOODSTUFF
PREPARATION PROCESSES

- 20 (iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:

- 25 (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:

- 30 (A) APPLICATION NUMBER: EP 92403582.7
(B) FILING DATE: 30-DEC-1992

(2) INFORMATION FOR SEQ ID NO: 1:

35 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3215 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- 45 (A) NAME/KEY: CDS
(B) LOCATION: 288..2663

(ix) FEATURE:

- 50 (A) NAME/KEY: CDS
(B) LOCATION: 288..2663

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

55 GATCTTAAAC TACAAGACTC CAGAAGAGTA CTTTGACACC GAACTTGACC GCATTTACCG 60
GCGTAGATAG TCAAAATCTG CCAGATGTGA TGGTAAGTGT TCAATTTATT CTTGCAATTG 120

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	GCGTTTTTTTT CTTGGTCATT TTTTCTTGA TCATTTTTTC CTTGACAAAA AGGAAGCGGT	180
	TTGCTTGCAT TAACTTGAGA ACTTGCTTAA AAAACAGCTA AAAAACTGG ATAAAGCAAA	240
5	AAGATACAAT ATCCCATAGA TGTAAAGATA TAAAGATGAG GTAAAGC ATG AAA TAC Met Lys Tyr 1	296
10	AAT CAA TAT GCC TAC GTT GAA ACC AGT CCG GAA AAG GCA ACT GAA GAG Asn Gln Tyr Ala Tyr Val Glu Thr Ser Pro Glu Lys Ala Thr Glu Glu 5 10 15	344
15	CTG CTG GCA ATC AAC TTC TTG CCG GAA AAC TAC TCT TCT CTT TCC TTC Leu Leu Ala Ile Asn Phe Leu Pro Glu Asn Tyr Ser Ser Leu Ser Phe 20 25 30 35	392
20	AGT GAG CTC CTG GCC GTC TTG ACC GGC AAT GTC CTG GCG GAA GCC ACG Ser Glu Leu Leu Ala Val Leu Thr Gly Asn Val Leu Ala Glu Ala Thr 40 45 50	440
25	ACC CGC CAG GCT AAA GAC GCA AAA TTG GCG GAA TTT GCC GTG GAC GAC Thr Arg Gln Ala Lys Asp Ala Lys Leu Ala Glu Phe Ala Val Asp Asp 55 60 65	488
30	CAA ACT GAC CTG GCC GCC TTT CTT TTG GAC ACG CCG ACT GCC ATC ACG Gln Thr Asp Leu Ala Ala Phe Leu Leu Asp Thr Pro Thr Ala Ile Thr 70 75 80	536
35	GCC AGC CAG TTC GCT AAT GTT GCC CTG CAG CTT TTA GGC TAC CAC CCA Ala Ser Gln Phe Ala Asn Val Ala Leu Gln Leu Leu Gly Tyr His Pro 85 90 95	584
40	AAC TAC GAC TAC AGC CTG ACT GAT CCT TTG ACT TGC GGG AAA AAG CAT Asn Tyr Asp Tyr Ser Leu Thr Asp Pro Leu Thr Cys Gly Lys Lys His 100 105 110 115	632
45	GCC CTG CCG GCC TTT AAG GAC TTG ACC AGC AAG GAA GAA TTG ATT TTT Ala Leu Pro Ala Phe Lys Asp Leu Thr Ser Lys Glu Glu Leu Ile Phe 120 125 130	680
50	ACC TTC TAC CGC CTG CTC AAC ACC CGG AGC AAA AAC GGG CAG ATC CTC Thr Phe Tyr Arg Leu Leu Asn Thr Arg Ser Lys Asn Gly Gln Ile Leu 135 140 145	728
55	TTG GAC GTC ATG GCC GGC AAG GGC TAC TTC ACC CAG TTC TGG GGG GAA Leu Asp Val Met Ala Gly Lys Gly Tyr Phe Thr Gln Phe Trp Gly Glu 150 155 160	776
60	GGC AAG TTT ATG TTC TTC AAC GGC AAG TCC CTG CCA GTT TTT GAC ACA Gly Lys Phe Met Phe Phe Asn Gly Lys Ser Leu Pro Val Phe Asp Thr 165 170 175	824
65	AGC CAG GTC ATC CGC GAG GTC GTT TAC GTG CAA AGC GAC CTG GAC ACG Ser Gln Val Ile Arg Glu Val Val Tyr Val Gln Ser Asp Leu Asp Thr 180 185 190 195	872

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	GAC GGC GAT GGC AAG GGC GAC CTC TTG CCG GTC ACG GTC TTC CGG CCA	920
	Asp Gly Asp Gly Lys Gly Asp Leu Leu Pro Val Thr Val Phe Arg Pro	
	200 205 210	
5	GTT GAA AGC CAG GAC CAG CTG AAG GTC CCA GCC CTC TAC ACG GCT TCA	968
	Val Glu Ser Gln Asp Gln Leu Lys Val Pro Ala Leu Tyr Thr Ala Ser	
	215 220 225	
10	CCT TAC TTT GGC GGG ATC ATC GAC AAT GTC AAG ACT AAC CAC AAT GTC	1016
	Pro Tyr Phe Gly Gly Ile Ile Asp Asn Val Lys Thr Asn His Asn Val	
	230 235 240	
15	GAC GAG AAC CTG ACT GAC GCG ACC ACT TGG ACC AAT CCC AAG TAC GTG	1064
	Asp Glu Asn Leu Thr Asp Ala Thr Thr Trp Thr Asn Pro Lys Tyr Val	
	245 250 255	
20	GCC AAG CCT TTA GTT AAA AGC CCT GCC CCC AGC GAC CAG GAC GTG CCG	1112
	Ala Lys Pro Leu Val Lys Ser Pro Ala Pro Ser Asp Gln Asp Val Pro	
	260 265 270 275	
	GCC ACT GAA CTG GCA ACT GGG CAA TCT TCT TAC GGC TTA AAC GAA TAC	1160
	Ala Thr Glu Leu Ala Thr Gly Gln Ser Ser Tyr Gly Leu Asn Glu Tyr	
	280 285 290	
25	CTC CTG GCC CGG GGC TTT GCC TCT GTC TTC TCC GGG GCC ATC GGC AAC	1208
	Leu Leu Ala Arg Gly Phe Ala Ser Val Phe Ser Gly Ala Ile Gly Asn	
	295 300 305	
30	CGG CAC GGG GAC GGG ATC CGG ATC ACC GGG TCA CCG GAA GAA ACC ATC	1256
	Arg His Gly Asp Gly Ile Arg Ile Thr Gly Ser Pro Glu Glu Thr Ile	
	310 315 320	
35	TCC CAA AAA GAA GTC ATT GAA TGG CTG ACT GGG GAC CGA GTG GCT TAT	1304
	Ser Gln Lys Glu Val Ile Glu Trp Leu Thr Gly Asp Arg Val Ala Tyr	
	325 330 335	
40	ACT GAC CGG ACC CGC CGC TTT GAA ACC AAA GCC AGC TGG TGC AGC GGC	1352
	Thr Asp Arg Thr Arg Arg Phe Glu Thr Lys Ala Ser Trp Cys Ser Gly	
	340 345 350 355	
	AAT GTC GGC ATG ACT GGC CGC TCT TAC CTG GGG ACC TTG CAG ATC GCC	1400
	Asn Val Gly Met Thr Gly Arg Ser Tyr Leu Gly Thr Leu Gln Ile Ala	
	360 365 370	
45	ATC GCG ACG ACC GGG GTC AAG GGC TTG AAG ACG GTG GTT TCT GAA GCC	1448
	Ile Ala Thr Thr Gly Val Lys Gly Leu Lys Thr Val Val Ser Glu Ala	
	375 380 385	
50	GCT ATT TCC TCT TGG TAT GAC TAC TAC CGG GAA CAC GGC CTG GTT GTC	1496
	Ala Ile Ser Ser Trp Tyr Asp Tyr Tyr Arg Glu His Gly Leu Val Val	
	390 395 400	
55	GCG CCA AGC GAA TGC CAG GGC GAA GAC ATG GAC AAG CTG GCT GAA GTC	1544
	Ala Pro Ser Glu Cys Gln Gly Glu Asp Met Asp Lys Leu Ala Glu Val	
	405 410 415	

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	TGC CAG TCC AAC CTC TGG GAC GGG GGC AAT TTC ACT GCT AAA AAG GCT	1592
	Cys Gln Ser Asn Leu Trp Asp Gly Gly Asn Phe Thr Ala Lys Lys Ala	
	420 425 430 435	
5	TAT GAA GCA GAG CAA GCT GAA CTT TTA GCC GCC CAG GAC CGG GCA ACC	1640
	Tyr Glu Ala Glu Gln Ala Glu Leu Leu Ala Ala Gln Asp Arg Ala Thr	
	440 445 450	
10	GGC CAG TAC TCT GAT TTC TGG GAG TCC AGA AAC TAC CGC CAC CAC ACC	1688
	Gly Gln Tyr Ser Asp Phe Trp Glu Ser Arg Asn Tyr Arg His His Thr	
	455 460 465	
15	GAT GGC ATC AAG TGC TCC TGG ATC TCC GTT CAC GGC TTA AAC GAC TGG	1736
	Asp Gly Ile Lys Cys Ser Trp Ile Ser Val His Gly Leu Asn Asp Trp	
	470 475 480	
	AAT GTC AAG CCG AAG AAC GTC TAC AAG ATC TGG CAA AAG GTC AAG CAG	1784
	Asn Val Lys Pro Lys Asn Val Tyr Lys Ile Trp Gln Lys Val Lys Gln	
	485 490 495	
20	CTG CCG GTG AAA TCT CAC CTC TTC CTC CAC CAG GGT CCG CAC TAC AAC	1832
	Leu Pro Val Lys Ser His Leu Phe Leu His Gln Gly Pro His Tyr Asn	
	500 505 510 515	
25	ATG AAC AAC CTG GTT TCC ATC GAT TTC ACC GAC CTC ATG AAC CTC TGG	1880
	Met Asn Asn Leu Val Ser Ile Asp Phe Thr Asp Leu Met Asn Leu Trp	
	520 525 530	
30	TTC GTC CAC GAG CTT TTG GAA GTG GAA AAC GGG GCT TAT GAG CAA TGG	1928
	Phe Val His Glu Leu Leu Glu Val Glu Asn Gly Ala Tyr Glu Gln Trp	
	535 540 545	
35	CCA AAG GTC ATG ATC CAA GAC AAC CTG GAA GCT GAC GAG TGG CAC GCA	1976
	Pro Lys Val Met Ile Gln Asp Asn Leu Glu Ala Asp Glu Trp His Ala	
	550 555 560	
40	GAA AGT GAC TGG GCC AGC GAC CTG GGC CAA GCC AGC CTC TAT TTA CCA	2024
	Glu Ser Asp Trp Ala Ser Asp Leu Gly Gln Ala Ser Leu Tyr Leu Pro	
	565 570 575	
45	ACG GCT GAC GGG GAC CTG TCA ACG GTT GAA AAC GGG ACA GGC CAG CTA	2072
	Thr Ala Asp Gly Asp Leu Ser Thr Val Glu Asn Gly Thr Gly Gln Leu	
	580 585 590 595	
50	ACC TTC ACC GAC TTG GGC GGG ACA GAA TTC AAG AAG GCC GGC ATT AGT	2120
	Thr Phe Thr Asp Leu Gly Gly Thr Glu Phe Lys Lys Ala Gly Ile Ser	
	600 605 610	
55	GAA ACT GAC TGG GAA TAC CAG TTT ATC AGC GGG GAG GAA AAA TGG GCC	2168
	Glu Thr Asp Trp Glu Tyr Gln Phe Ile Ser Gly Glu Glu Lys Trp Ala	
	615 620 625	
55	AAG GCC AGC CTC CGC TTT GAA AGC GAG GAA TTC CTC CAC CCG ACT ACC	2216
	Lys Ala Ser Leu Arg Phe Glu Ser Glu Glu Phe Leu His Pro Thr Thr	
	630 635 640	

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	CTG GTC GGC CGG CCA AAG GTC CGG GTC CGG GTA GCA GCT AAT AAG ACT	2264
	Leu Val Gly Arg Pro Lys Val Arg Val Arg Val Ala Ala Asn Lys Thr	
	645 650 655	
5	GTC GGC CAG CTG TCT GTT GCC CTG GTT GAC CTG GGG ACC CGG CAG CGG	2312
	Val Gly Gln Leu Ser Val Ala Leu Val Asp Leu Gly Thr Arg Gln Arg	
	660 665 670 675	
10	CTG ACG GCT ACG CCG AAG ATT TTT GCC AGA GGA AAC CAG CCC TTT GGC	2360
	Leu Thr Ala Thr Pro Lys Ile Phe Ala Arg Gly Asn Gln Pro Phe Gly	
	680 685 690	
15	TAC CGC TTC GGG GCA GAC TCC TTG CAG GAA TTC GTC CCG GAC AAG GCC	2408
	Tyr Arg Phe Gly Ala Asp Ser Leu Gln Glu Phe Val Pro Asp Lys Ala	
	695 700 705	
20	ACT AAG GCC AAG CTG ATT ACC AAG GCC CAC ATG AAC CTG CAG AAC TAC	2456
	Thr Lys Ala Lys Leu Ile Thr Lys Ala His Met Asn Leu Gln Asn Tyr	
	710 715 720	
	CAG GAC ATG AAG CAG CCA AGC AAG CTG GAA GCC GGC CAG TTC GTC GAC	2504
	Gln Asp Met Lys Gln Pro Ser Lys Leu Glu Ala Gly Gln Phe Val Asp	
	725 730 735	
25	CTA GAA TTT GAA CTG CAG CCG ACC TAC TAC ACC CTG CCA GCT GGA GCA	2552
	Leu Glu Phe Glu Leu Gln Pro Thr Tyr Thr Thr Leu Pro Ala Gly Ala	
	740 745 750 755	
30	AAA TTG GGT CTG ATC ATC TAT TCA ACC GAC CAG GGG ATG ACC AAG CGG	2600
	Lys Leu Gly Leu Ile Ile Tyr Ser Thr Asp Gln Gly Met Thr Lys Arg	
	760 765 770	
35	CCG CTG GAG ACC GAA GAC TAC ACA GTT GAC TTA GCC GGA ACT GCT CTT	2648
	Pro Leu Glu Thr Glu Asp Tyr Thr Val Asp Leu Ala Gly Thr Ala Leu	
	775 780 785	
40	TTG CTC TAC CGG AAA TAATGCCTGG GCAAAAATTG GCGGTTTATG CTAAAATAAC	2703
	Leu Leu Tyr Arg Lys	
	790	
	TTTATCGCAA ACAAAGTAAA AGGCTGTGCG GGACGTACCG CTGACAGCCT TTTTGTGTTT	2763
	TTAGAAAGGT CCTTATGGAA AAATCAAGCA AAGTCAACCT TGCTATTTTC AGTGGGGGCC	2823
45	TGATGACCTT CATCGGTATT CTGAATGAAA CCTCCATGAA CGTCACCTAC CCGCTTTTAG	2883
	TCAAAGAGTT CCACCAGCCC CTGGCAACCG TCCAGGTAGA TCACTACCGC CTATCTCTTG	2943
50	ACAGTGACCA TCGTCATGGG AACTACCGCC TACCTGCTCA AGCAGGTCCC GGCTCGCTGG	3003
	CTGCATTTAG GAGCCACCCT GGCCTTTATT TGCGGTTGCA TCATCTGCGC CTTAACTTCC	3063
	AGCTTTCCTT TGATGCTGGC CGGCCGGATC ATTCAGGGGA TCGCCACCGG CTTTTCCACC	3123
55	CCGATCATGT TCCAGCTGAT CTTTACCCAG GTCCCTAAGC AAAAACTCGG CCTCATGACC	3183
	GGTTTTGCCG GGACGATCAT CTCCTTTGCC CC	3215

- 27 -

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 792 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

10

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

Met Lys Tyr Asn Gln Tyr Ala Tyr Val Glu Thr Ser Pro Glu Lys Ala
 1 5 10 15

Thr Glu Glu Leu Leu Ala Ile Asn Phe Leu Pro Glu Asn Tyr Ser Ser
 20 25 30

Leu Ser Phe Ser Glu Leu Leu Ala Val Leu Thr Gly Asn Val Leu Ala
 35 40 45

Glu Ala Thr Thr Arg Gln Ala Lys Asp Ala Lys Leu Ala Glu Phe Ala
 50 55 60

Val Asp Asp Gln Thr Asp Leu Ala Ala Phe Leu Leu Asp Thr Pro Thr
 65 70 75 80

Ala Ile Thr Ala Ser Gln Phe Ala Asn Val Ala Leu Gln Leu Leu Gly
 85 90 95

Tyr His Pro Asn Tyr Asp Tyr Ser Leu Thr Asp Pro Leu Thr Cys Gly
 100 105 110

Lys Lys His Ala Leu Pro Ala Phe Lys Asp Leu Thr Ser Lys Glu Glu
 115 120 125

Leu Ile Phe Thr Phe Tyr Arg Leu Leu Asn Thr Arg Ser Lys Asn Gly
 130 135 140

Gln Ile Leu Leu Asp Val Met Ala Gly Lys Gly Tyr Phe Thr Gln Phe
 145 150 155 160

Trp Gly Glu Gly Lys Phe Met Phe Phe Asn Gly Lys Ser Leu Pro Val
 165 170 175

Phe Asp Thr Ser Gln Val Ile Arg Glu Val Val Tyr Val Gln Ser Asp
 180 185 190

Leu Asp Thr Asp Gly Asp Gly Lys Gly Asp Leu Leu Pro Val Thr Val
 195 200 205

Phe Arg Pro Val Glu Ser Gln Asp Gln Leu Lys Val Pro Ala Leu Tyr
 210 215 220

Thr Ala Ser Pro Tyr Phe Gly Gly Ile Ile Asp Asn Val Lys Thr Asn
 225 230 235 240

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	His	Asn	Val	Asp	Glu	Asn	Leu	Thr	Asp	Ala	Thr	Thr	Trp	Thr	Asn	Pro	
					245					250					255		
5	Lys	Tyr	Val	Ala	Lys	Pro	Leu	Val	Lys	Ser	Pro	Ala	Pro	Ser	Asp	Gln	
				260					265					270			
	Asp	Val	Pro	Ala	Thr	Glu	Leu	Ala	Thr	Gly	Gln	Ser	Ser	Tyr	Gly	Leu	
			275					280					285				
10	Asn	Glu	Tyr	Leu	Leu	Ala	Arg	Gly	Phe	Ala	Ser	Val	Phe	Ser	Gly	Ala	
		290					295					300					
	Ile	Gly	Asn	Arg	His	Gly	Asp	Gly	Ile	Arg	Ile	Thr	Gly	Ser	Pro	Glu	
15	305					310					315					320	
	Glu	Thr	Ile	Ser	Gln	Lys	Glu	Val	Ile	Glu	Trp	Leu	Thr	Gly	Asp	Arg	
					325					330					335		
20	Val	Ala	Tyr	Thr	Asp	Arg	Thr	Arg	Arg	Phe	Glu	Thr	Lys	Ala	Ser	Trp	
				340					345					350			
	Cys	Ser	Gly	Asn	Val	Gly	Met	Thr	Gly	Arg	Ser	Tyr	Leu	Gly	Thr	Leu	
			355					360					365				
25	Gln	Ile	Ala	Ile	Ala	Thr	Thr	Gly	Val	Lys	Gly	Leu	Lys	Thr	Val	Val	
		370					375					380					
	Ser	Glu	Ala	Ala	Ile	Ser	Ser	Trp	Tyr	Asp	Tyr	Tyr	Arg	Glu	His	Gly	
30	385					390					395					400	
	Leu	Val	Val	Ala	Pro	Ser	Glu	Cys	Gln	Gly	Glu	Asp	Met	Asp	Lys	Leu	
				405						410					415		
	Ala	Glu	Val	Cys	Gln	Ser	Asn	Leu	Trp	Asp	Gly	Gly	Asn	Phe	Thr	Ala	
35				420					425					430			
	Lys	Lys	Ala	Tyr	Glu	Ala	Glu	Gln	Ala	Glu	Leu	Leu	Ala	Ala	Gln	Asp	
			435					440					445				
40	Arg	Ala	Thr	Gly	Gln	Tyr	Ser	Asp	Phe	Trp	Glu	Ser	Arg	Asn	Tyr	Arg	
		450					455					460					
	His	His	Thr	Asp	Gly	Ile	Lys	Cys	Ser	Trp	Ile	Ser	Val	His	Gly	Leu	
45	465					470					475					480	
	Asn	Asp	Trp	Asn	Val	Lys	Pro	Lys	Asn	Val	Tyr	Lys	Ile	Trp	Gln	Lys	
					485					490					495		
	Val	Lys	Gln	Leu	Pro	Val	Lys	Ser	His	Leu	Phe	Leu	His	Gln	Gly	Pro	
50				500					505					510			
	His	Tyr	Asn	Met	Asn	Asn	Leu	Val	Ser	Ile	Asp	Phe	Thr	Asp	Leu	Met	
			515					520					525				
55	Asn	Leu	Trp	Phe	Val	His	Glu	Leu	Leu	Glu	Val	Glu	Asn	Gly	Ala	Tyr	
		530					535					540					

[illegible]

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CLAIMS

1. Protein in a substantially pure form, as produced and excreted by *Lactobacillus delbrückii* ssp. *lactis* and capable of releasing proline-containing dipeptides (or X-Pro dipeptides) from the N-terminus of oligopeptides present in the culture medium of said lactobacilli, or fragments thereof having said releasing X-Pro dipeptides activity.

2. Protein according to claim 1, such as produced by *Lactobacillus delbrückii* ssp. *lactis* WS87, deposited at the Deutsche Sammlung von Mikroorganismen (DSM) under the number 7290 on October 15, 1992.

3. Protein according to claim 1 or claim 2, characterized in that it corresponds to SEQ ID NO 2 (also represented on figure 5 or figure 6), or fragments or muteins (which differ from said protein by addition and/or substitution and/or suppression of one or several amino acid) thereof, provided that said fragments and muteins are capable of releasing X-Pro dipeptides.

4. Protein according to claim 3, characterized in that:

- its isoelectric point calculated from the nucleotide sequence represented by SEQ ID NO 2, is 4.96, and its isoelectric point determined by preparative isoelectric focusing after ammonium sulfate fractionation of crude extract of said protein, is 3.9,

- its molecular weight calculated from the nucleotide sequence represented by SEQ ID NO 2, is 88.449 kDa, and its molecular weights determined by SDS-PAGE after purification, by SDS-PAGE after in vitro translation of plasmid pJK433, and by gel filtration, are 95 kDa, 89.9 kDa and 84 kDa respectively,

- its hydropathy profile is represented on figure 8,

- specific chromogenic substrates of said PepX are chosen among L-Ala-L-Pro-p-nitroanilide and Gly-L-Pro- β -naphthylamide,

- specific inhibitors of said PepX are the following:

. phenylmethanesulfonylfluoride (PMSF), end concentration 1 mM: 73 % relative activity,

. 3,4-dichloroisocoumarin (3,4-DCI): end concentration 0,1 mM: 55 % relative activity,

. 4-(2-aminoethyl)-phenylsulfonylfluoride, hydrochloride (AEBSF), end concentration 0,1 mM: 92 % relative activity.

5. Protein according to any one of claims 1 to 4, such as obtained from a cell extract of *Lactobacillus delbrückii* ssp. *lactis*, by the following four-step procedure:

- fractionation of the cell extract by salting out at 4°C with solid ammonium sulfate from 40 to 60 % saturation,
- hydrophobic interaction chromatography by applying the ammonium sulfate fraction obtained at the previous step to an appropriate column such as a column of Octyl-Sepharose 4B (Pharmacia), and pooling the eluted fractions having the highest specific activity against L-Ala-L-Pro-p-nitroanilide,
- ion exchange chromatography, by applying the pooled fractions obtained at the previous step to an appropriate column such as a Mono Q HR (Pharmacia),
- affinity chromatography using L-Ala-L-Pro linked to EAH-Sepharose 4B (Pharmacia) as ligand.

6. Nucleic acid characterized in that:

- it comprises all or part of the nucleic acid represented by SEQ ID NO 1 (and also on figure 5 or figure 6), coding for a protein according to anyone of claims 1 to 5, or its complementary sequence,
- or it hybridizes with all or part of said nucleic acid represented by SEQ ID NO 1, or with its complementary sequence.

7. Recombinant nucleic acid containing at least one of the nucleic acids according to claim 6, inserted in a heterologous nucleic acid.

8. Recombinant vector comprising a vector sequence, notably of the type plasmid (such as plasmids originating from lactic acid bacteria), cosmid or phage, and a nucleic acid according to anyone of claims 6 or 7, in one of the non-essential sites for its replication.

9. Recombinant vector according to claim 8, containing in one of its non essential sites for its replication necessary elements to promote the expression of polypeptides according to anyone of claims 1 to 5 in a cellular host and possibly a promoter recognized by the polymerase of the cellular host, particularly an inducible promoter and possibly a signal sequence and/or an anchoring sequence.

10. Recombinant vector according to claim 9, containing the elements enabling the expression by E. coli or lactic acid bacteria including *lactococcus* and, especially, lactobacilli of the thermophilic group, of a nucleic acid according to anyone of claims 6 or 7, inserted in the vectors.

5

11. Recombinant vector according to claim 10 or 11, constructed from replicons isolated from lactobacilli, especially from thermophilic *Lactobacillus* species.

10

12. Cellular host which is transformed by a recombinant vector according to anyone of claims 8 to 11, and comprising the regulation elements enabling the expression of the nucleotide sequence coding for a protein according to anyone of claims 1 to 5 in this host.

15

13. Cellular host according to claim 12, chosen from among bacteria such as E.coli, or lactic acid bacteria including *lactococcus* and, especially, lactobacilli of the thermophilic group, transformed by a vector according to anyone of claims 8 to 11.

20

14. Expression product of a nucleic acid expressed by a transformed cellular host according to anyone of claims 12 or 13.

15. Process for preparing a protein according to anyone of claims 1 to 5 comprising the following steps:

25

- the culture in an appropriate medium of a cellular host according to anyone of claims 12 or 13,

- the recovery of the polypeptide produced by the above said cellular host from the above said culture medium,

- the purification of the protein thus obtained, more particularly according to the method described in claim 5.

30

16. Fermentation process characterized in that it comprises a step of treatment of material to be fermented with:

35

- an appropriate amount of at least one of the protein according to claims 1 to 5, and/or

- an appropriate amount of at least one of the transformed cellular host according to anyone of claims 12 or 13, and/or

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- an appropriate amount of lactobacilli, such as *Lactobacillus delbrückii* ssp. *lactis*.

17. Process for the preparation of fermented foodstuff, and more particularly of cheese, which comprises a step of treatment of food material to be fermented, such as milk, with:

- an appropriate amount of at least one of the protein according to claims 1 to 5, and/or

- an appropriate amount of at least one of the transformed cellular host according to anyone of claims 12 or 13, and/or

- an appropriate amount of lactobacilli, such as *Lactobacillus delbrückii* ssp. *lactis*.

18. Process according to claim 17, characterized in that it also comprises a step of treatment of food material to be fermented, such as milk, with other species and strains susceptible to be used as starter organisms in fermentation processes, and more particularly lactic acid bacteria susceptible to produce a PepX protein, such as *Lactococcus*, *Streptococcus* and *Lactobacillus*.

19. Process according to claim 17 or claim 18, characterized in that it can be used as fermentation processes for the obtention of hard cheeses, such as Emmentaler type cheese.

20. Foodstuff, and more particularly cheese, such as obtained by the method according to any one of claims 17 to 19.

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1 2 3 4 5 6

a

b

c

d

e

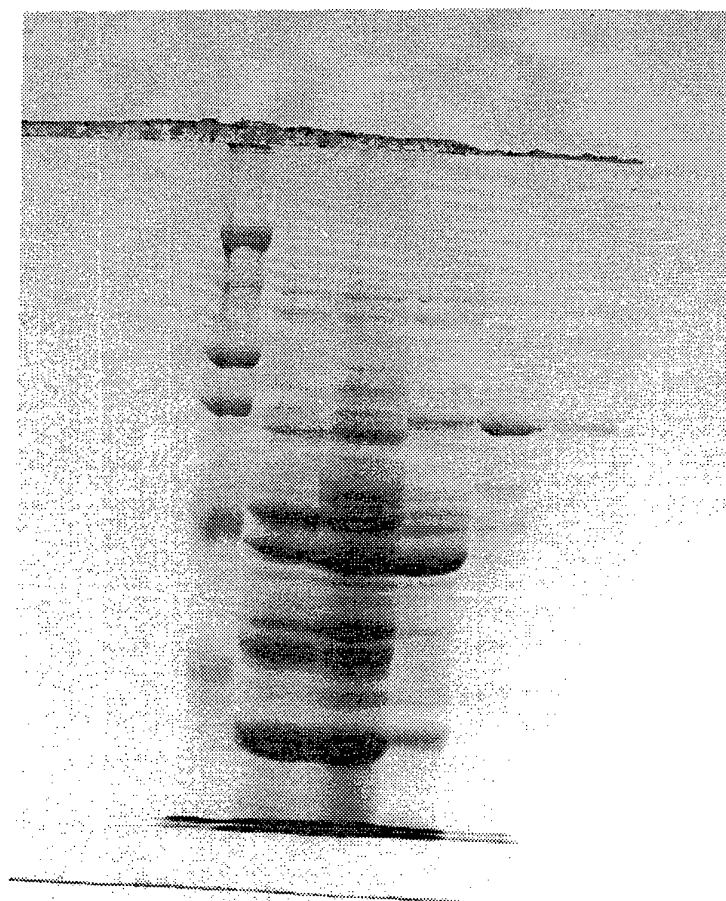


Figure 1

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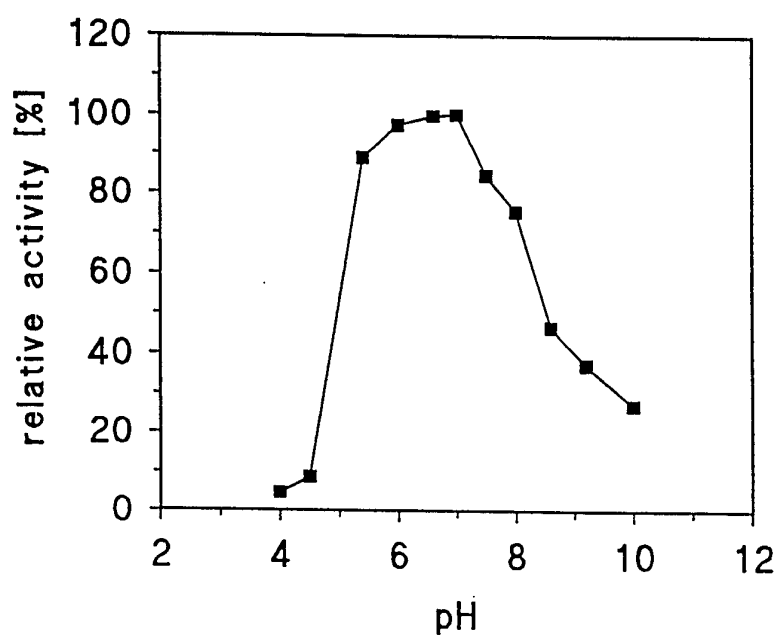
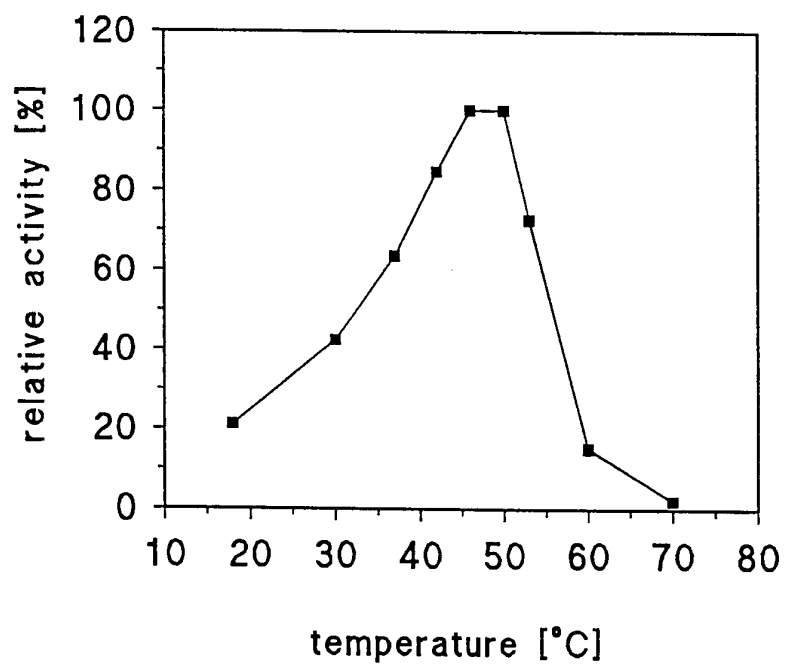


Figure 2 (a and b)
SUBSTITUTE SHEET

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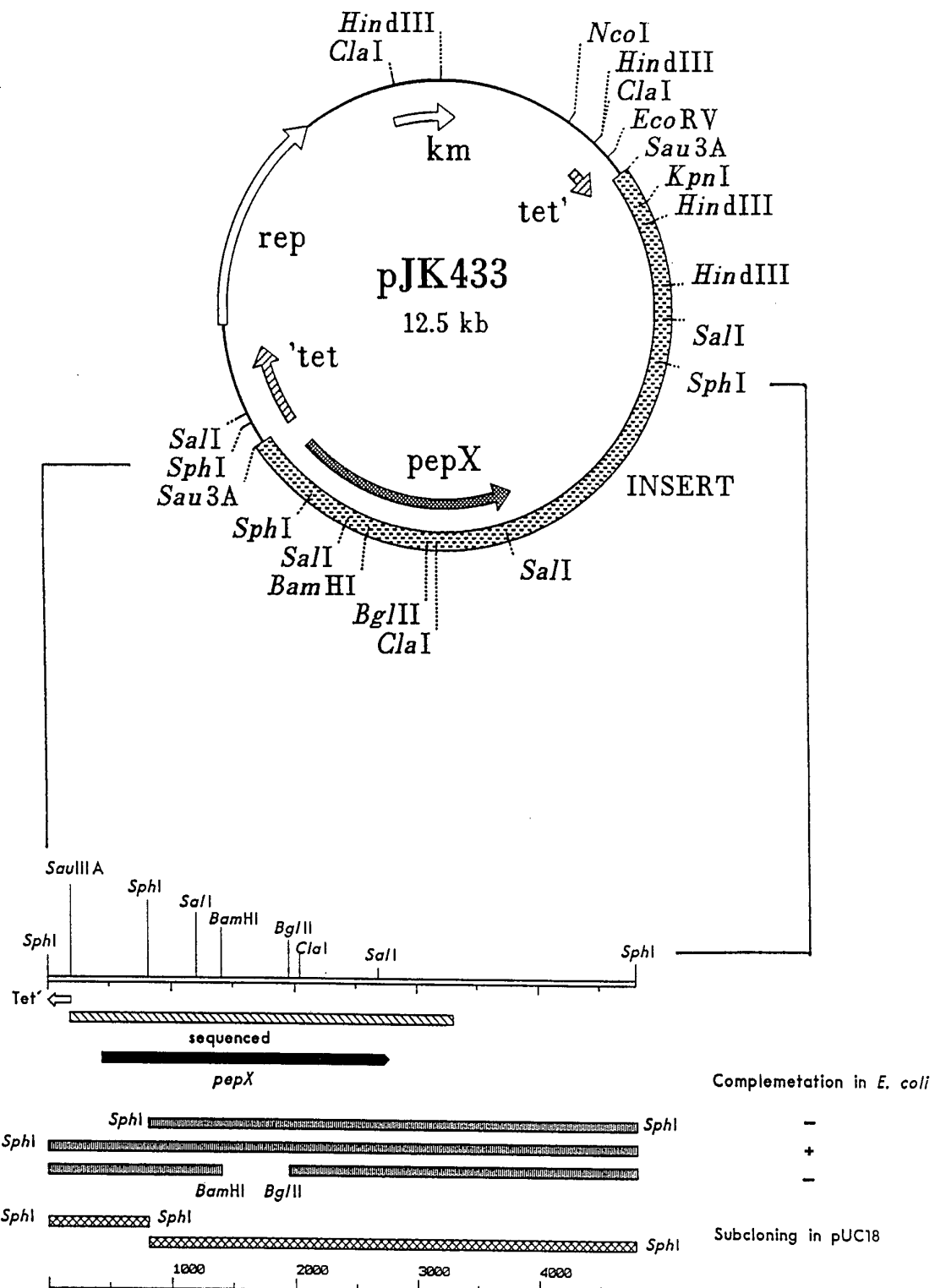


Figure 3
SUBSTITUTE SHEET

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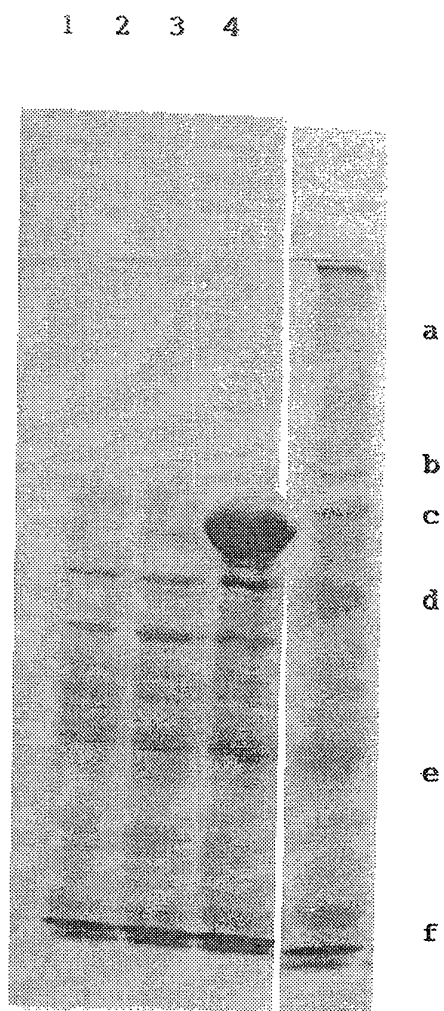


Figure 4

Figure 5

SauIIIA 10 20 30 40 50 60 70 80 90 100
GATCTTAAACTACAAGACTCCAGAAGAGTACTTTGACACCGCACTTGACCGGTAGATAGTCAAAATCTGCCAGATGTTATGGTAAAGTGT
 110 120 130 140 150 160 170 180 190 200
 TCAATTTATCTTGCAATTGGCGTTTTTTTCTTGGTCAATTTTTTCTTGATCATTTTTTCCCTTGACAAAAGGAAGCGGTTTGCTTGCAATAACTTGAGA
 (-35)
 pepX -->
 210 220 230 240 250 260 270 280 290 300
 ACTTGCTTAAAAAACAGCTAAAAAACTGGATAAAGCAAAAAGATACAATATCCCATAGATGTAAAGATATAAAGATGAGGTAAAGCATGAATAACAATC
 (-10) M K Y N
 310 320 330 340 350 360 370 380 390 400
 AATATGCCCTACGTTGAAACCAGTCCGGAAAGGCAACTGAAGAGCTGCTGGCAATCAACTTCTTGCCGGAAAACACTACTCTTCTCTTCTTCCCTTCAGTGAGCT
 Q Y A Y V E T S P E K A T E E L L A I N F L P E N Y S S L S F S E L
 410 420 430 440 450 460 470 480 490 500
 CCTGGCCGTCTTGACCGGCAATGTCCTGCGGAAGCCACGACCCGCCAGGCTAAAGACGCAAAATTTGCCGTGGACGACCAAACTGACCTG
 L A V L T G N V L A E A T T R Q A K D A K L A E F A V D D Q T D L
 510 520 530 540 550 560 570 580 590 600
 GCCGCCCTTTTGGACACGCCGACTGCCATCACGGCCAGCCAGTTTCGCTAATGTTGCCCTGCAGCTTTTAGGCTACCAACCAAACTACGACTACAGCC
 A A F L L D T P T A I T A S Q F A N V A L Q L L G Y H P N Y D Y S
 610 620 630SphI 640 650 660 670 680 690 700
 TGACTGATCCTTTGACTTGCGGGGAAAAAGCATGCCCTGCCGGCCTTTTAAAGGACTTGACCCAGCAAGGAAGAAATTTTACCTTTTACCTTCTACCGCCTGCTCAA
 L T D P L T C G K K H A L P A F K K D L T S K E E L I F T F Y R L L N
 710 720 730 740 750 760 770 780 790 800
 CACCCGGAGCAAAAACGGGCAGATCCTCTTGACGTCATGGCCGCGCAAGGGCTACTTCACCCAGTTCTGGGGGAAGGCAAGTTATGTTCTTCAACGGC
 T R S K N G Q I L L D V M A G K G Y F T Q F W G E G K F M F F N G
 810 820 830 840 850 860 870 880 890 900
 AAGTCCCTGCCAGTTTGTGACACAAGCCAGGTATCCCGGAGGTTCGTTTACGTGCAAGCGACCTGGACACGGCGGATGGCAAGGGCGGACCTCTTGC
 K S L P V F D T S Q V I R E V V Y V Q S D L D T D G D G K G D L L

Figure 5 (cont. 1)

910	920	930	940	950	960	970	980	990	1000
CGGTACGGTCTTCCGGCCAGTTGAAAGCCAGGACCAAGCTGAAGTCCAGCCCTCTACACGGCTTACCTTACTTTGGCGGGATCATCGACAATGTCAA	P V T V F R P V E S Q D Q L K V P A L Y T A S P Y F G G I I D N V K								
1010	1020	1030	1040	1050	1060	1070	1080	1090	1100
GACTAACCAACAATGTCGACGAGAACCTGACTGACGGGACCACTTGGACCAATCCCAAGTACGTTGGCCCAAGCCTTTAGTTAAAGCCCTGCCCCCAGCGAC	T N H N V D E N L T D A T T W T N P K Y V A K P L V K S P A P S D								
1110	1120	1130	1140	1150	1160	1170	1180	1190	1200
CAGGACGTCCGGCCCACTGAACCTGGCAACTGCGCAATCTTCTTACGGCTTAAACGAATACCTCCTGGCCCGGGCTTTGCCCTCTGTCTTCTCCGGGGCCA	Q D V P A T E L A T G Q S S Y G L N E Y L L A R G F A S V F S G A								
1210	1220	BamHI1230	1240	1250	1260	1270	1280	1290	1300
TCGGCAACCGGCACGGGACGGGATCCGGATCACCGGGTCACCGGAAGAAACCATCTCCCAAAAAGATCATTTGAATGGCTGACTGGGGACCGAGTGGC	I G N R H G D G I R I T G S P E E T I S Q K E V I E W L T G D R V A								
1310	1320	1330	1340	1350	1360	1370	1380	1390	1400
TTATACTGACCGGACCGCGCTTTGAAACCAAAAGCCAGCTGGTGCAGCGGCAATGTGGCATGACTGGCCGCTCTTACCTGGGGACCTTGCAGATCGCC	Y T D R T R R F E T K A S W C S G N V G M T G R S Y L G T L Q I A								
1410	1420	1430	1440	1450	1460	1470	1480	1490	1500
ATCGCGACGACCGGGGTCAAGGGCTTGAAGACGGTGGTTCTGAAGCCGCTATTTCCTCTTGGTATGACTACTACCGGGAACACACGGCCCTGGTGTCTCGCGC	I A T T G V K G L K T V V S E A A I S S W Y D Y Y R E H G L V V A								
1510	1520	1530	1540	1550	1560	1570	1580	1590	1600
CAAGCGAATGCCAGGCGAAGACATGGACAAGCTGGCTGAAGTCTGCCAGTCCAACTCTGGGACGGGGGCAATTTCACTGCTATAAAGGCTTATGAAGC	P S E C Q G E D M D K L A E V C Q S N L W D G G N F T A K K A Y E A								
1610	1620	1630	1640	1650	1660	1670	1680	1690	1700
AGAGCAAGCTGAACCTTTAGCCGCCAGGACCGGGCAACCGGCCAGTACTCTGATTTCTGGGAGTCCAGAAACTACCGCCACACACCGATGGCATCAAG	E Q A E L L A A Q D R A T G Q Y S D F W E S R N Y R H H T D G I K								
1710	1720	1730	1740	1750	1760	BglIII1770	1780	1790	1800
TGCTCCTGGATCTCCGTTACGGCTTAAACGACTGGAATGTCAAGCCGGAAGAACGTTCTACAAAGATCTGGCAAAAGGTCAAGCAGCTGCCGGTGAATCTC	C S W I S V H G L N D W N V K P K N V Y K I W Q K V K Q L P V K S								

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Figure 5 (cont. 2)

1810 1820 1830 1840 1850 Clal 1860 1870 1880 1890 1900
 ACCTCTTCCTCCACGAGGTCCGCACTACAACATGAACAACCTGGTTTCCATCGATTTCAACCGACCTCATGAACCTCTGGTTCCGTCACGAGCTTTTGA
 H L F L H Q G P H Y N M N N L V S I D F T D L M N L W F V H E L L E

 1910 1920 1930 1940 1950 1960 1970 1980 1990 2000
 AGTGGAAAACGGGGCTTATGAGCAATGGCCAAAGGTGATCATGATCCAAGCAACCTGGAGCTGACGAGTGGCAGCAGAAAGTGAAGTGGCCAGCGACCTG
 V E N G A Y E Q W P K V M I Q D N L E A D E W H A E S D W A S D L

 2010 2020 2030 2040 2050 2060 2070 2080 2090 2100
 GGCCAAGCCAGCCTCTATTTACCAACGGCTGACGGGACCTGTCAACGGTTGAAAACGGGACAGGCCAGCTAACCTTCACCGACTTGGCGGGACAGAAAT
 G Q A S L Y L P T A D G D L S T V E N G T G Q L T F T D L G G T E

 2110 2120 2130 2140 2150 2160 2170 2180 2190 2200
 TCAAGAAGCCGGCATTAGTGAACTGACTGGGAATACCAAGTTTATCAGCGGGGAGGAAAATGGGCCAAGGCCAGCCTCCGCTTTGAAAAGCGAGGAATT
 F K K A G I S E T D W E Y Q F I S G E E K W A K A S L R F E S E E F

 2210 2220 2230 2240 2250 2260 2270 2280 2290 2300
 CCTCCACCGACTACCTGGTCCGGCCGCAAGGTCCGGGTCCGGGTAGCAGCTAATAAGACTGTGCGCCAGCTGTCTGTGTCCTGCTGTTGACCTGGGG
 L H P T T L V G R P K V R V A A N K T V G Q L S V A L V D L G

 2310 2320 2330 2340 2350 2360 2370 2380 2390 2400
 ACCCGGCAGCGGTGACGGCTACGGCCGAAGATTTTGGCCAGAGGAAACCAAGCCCTTTGGCTACCGCTTCGGGGCAGACTCCTTGCAGGAATTTCGTCCCGG
 T R Q R L T A T P K I F A R G N Q P F G Y R F G A D S L Q E F V P

 2410 2420 2430 2440 2450 2460 2470 2480 2490 2500
 ACAAGGCCACTAAGCCAAAGCTGATTACCAAGGCCCACTGACATGAACCTGCAGAACTACCAAGGACATGAAGCAAGCTGGAAGCCGCGCAGTTCTGT
 D K A T K A K A K L I T K A H M N L Q N Y Q D M K Q P S K L E A G Q F V

 2510 2520 2530 2540 2550 2560 2570 2580 2590 2600
 CGACCTAGAATTGAACTGCAGCCGACCTACTACACCTGCCAGCTGGAGCAAAATTTGGGTCTGATCATCTATTCAACCGACCGGGGATGACCAAGCGG
 D L E F E L Q P T Y Y T L P A G A K L G L I I Y S T D Q G M T K R

 2610 2620 2630 2640 2650 2660 2670 2680 2690 2700
 CCGCTGGAGACCGGAAGACTACACAGTTGACTTAGCCGGAACCTGCTCTTTTGTCTCTACCGGAAATAATGCTGGGCAAAATTTGGCGGTTTATGCTAAAT
 P L E T E D Y T V D L A G T A L L L Y R K *

Figure 5 (cont. 3)

2710 2720 2730 2740 2750 2760 2770 2780 2790 2800
AACTTTATCGCAACAAGTAAAGGCTGTGCGGACGTACCGCTGACAGCCTTTTGTGTTCTTTAGAAAGGTCTTATGGAAAAATCAAGCAAAAGTCAA
-----><-----
2810 2820 2830 2840 2850 2860 2870 2880 2890 2900
CCTTGCTATTTTCAGTGGGGCCGTGATGACCTTCATCGGTATTCTGAATGAAACCTCCATGAACGTCACTACCCGCTTTTAGTCAAAAGAGTTCCACCAG
2910 2920 2930 2940 2950 2960 2970 2980 2990 3000
CCCCTGGCAACCGTCCAGGTAGATCACTACCGCCTATCTCTTGACAGTGACCAATCGTCAATGGGAACTACCGCCTACCTGCTCAAGCAGGTCCCGGCTCGC
3010 3020 3030 3040 3050 3060 3070 3080 3090 3100
TGGCTGCATTTAGGAGCCACCCGTGGCCCTTATTGCGGTTGCAATCATCTGGCCCTTAACTTCCAGCTTTCCCTTGATGCTGGCCCGCGGATCATTCAGG
3110 3120 3130 3140 3150 3160 3170 3180 3190 3200
GGATCGCCACCGGCTTTTCCACCCCGATCATGTTCAGCTGATCTTTACCCAGGTCCCTAAGCAAAAACCTCGGCCCTCATGACCGGTTTGTCCCGGACGAT
3210 3220 3230 3240 3250 3260 3270 3280 3290 3300
CATCTCCTTTGCCCC

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GATCTTAAACTACAAGACTCCAGAAGAGTACTTTGACACCGAACTTGACCGCATTTACCG
GCGTAGATAGTCAAAATCTGCCAGATGTTATGGTAAGTGTTCAATTTATTCTTGCAATTG
GCGTTTTTTTTCTTGGTCATTTTTTTCTTGATCATTTTTTCCTTGACAAAAAGGAAGCGGT
TTGCTTGCATTAACTTGAGAACTTGCTTAAAAAACAGCTAAAAAACTGGATAAAGCAAA
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MetLysTyrAsnG
AATATGCCTACGTTGAAACCACTCCGGAAGGCAACTGAAGAGCTGCTGGCAATCAACT
lnTyrAlaTyrValGluThrSerProGluLysAlaThrGluGluLeuLeuAlaIleAsnP
TCTTGCCGGAAGCTACTCTTCTTTCTTCCTTCAGTGAGCTCCTGGCCGTCTTGACCGGCA
heLeuProGluAsnTyrSerSerLeuSerPheSerGluLeuLeuAlaValLeuThrGlyA
ATGTCCTGGCGGAAGCCACGACCCGCCAGGCTAAAGACGCAAAATTGGCGGAATTTGCCG
snValLeuAlaGluAlaThrThrArgGlnAlaLysAspAlaLysLeuAlaGluPheAlaV
TGGACGACCAAACTGACCTGGCCGCCTTTCTTTTGGACACGCCGACTGCCATCACGGCCA
alAspAspGlnThrAspLeuAlaAlaPheLeuLeuAspThrProThrAlaIleThrAlaS
GCCAGTTCGCTAATGTTGCCCTGCAGCTTTTAGGCTACCACCCAACTACGACTACAGCC
erGlnPheAlaAsnValAlaLeuGlnLeuLeuGlyTyrHisProAsnTyrAspTyrSerL
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euThrAspProLeuThrCysGlyLysLysHisAlaLeuProAlaPheLysAspLeuThrS
GCAAGGAAGAATTGATTTTTACCTTCTACCGCTGCTCAACACCCGGAGCAAAAACGGGC
erLysGluGluLeuIlePheThrPheTyrArgLeuLeuAsnThrArgSerLysAsnGlyG
AGATCCTCTTGACGTCATGGCCGGCAAGGGCTACTTCACCCAGTTCTGGGGGGAAGGCA
lnIleLeuLeuAspValMetAlaGlyLysGlyTyrPheThrGlnPheTrpGlyGluGlyL
AGTTTATGTTCTTCAACGGCAAGTCCCTGCCAGTTTTTGACACAAGCCAGGTCATCCGCG
ysPheMetPhePheAsnGlyLysSerLeuProValPheAspThrSerGlnValIleArgG
AGGTCGTTTACGTGCAAAGCGACCTGGACACGGACGGCGATGGCAAGGGCGACCTCTTGC
luValValTyrValGlnSerAspLeuAspThrAspGlyAspGlyLysGlyAspLeuLeuP
CGGTCACGGTCTTCCGGCCAGTTGAAAGCCAGGACCAGCTGAAGGTCCCAGCCCTCTACA
roValThrValPheArgProValGluSerGlnAspGlnLeuLysValProAlaLeuTyrT
CGGCTTACCTTACTTTGGCGGGATCATCGACAATGTCAAGACTAACCACAATGTCGACG
hrAlaSerProTyrPheGlyGlyIleIleAspAsnValLysThrAsnHisAsnValAspG
AGAACCTGACTGACGCGACCACTTGGACCAATCCCAAGTACGTGGCCAAGCCTTTAGTTA
luAsnLeuThrAspAlaThrThrTrpThrAsnProLysTyrValAlaLysProLeuValL
AAAGCCCTGCCCCAGCGACAGGACGTGCCGGCCACTGAACTGGCAACTGGGCAATCTT
ysSerProAlaProSerAspGlnAspValProAlaThrGluLeuAlaThrGlyGlnSerS

Figure 6

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CTTACGGCTTAAACGAATACCTCCTGGCCCCGGGGCTTTGCCTCTGTCTTCTCCGGGGCCA
erTyrGlyLeuAsnGluTyrLeuLeuAlaArgGlyPheAlaSerValPheSerGlyAlaI
TCGGCAACCGGCACGGGGACGGGATCCGGATCACCGGGTCACCGGAAGAAACCATCTCCC
leGlyAsnArgHisGlyAspGlyIleArgIleThrGlySerProGluGluThrIleSerG
AAAAAGAAGTCATTGAATGGCTGACTGGGGACCGAGTGGCTTATACTGACCGGACCCGCC
lnLysGluValIleGluTrpLeuThrGlyAspArgValAlaTyrThrAspArgThrArgA
GCTTTGAAACCAAAGCCAGCTGGTGCAGCGGCAATGTCGGCATGACTGGCCGCTCTTACC
rgPheGluThrLysAlaSerTrpCysSerGlyAsnValGlyMetThrGlyArgSerTyrL
TGGGGACCTTGCAGATCGCCATCGCGACGACCGGGGTCAAGGGCTTGAAGACGGTGGTTT
euGlyThrLeuGlnIleAlaIleAlaThrThrGlyValLysGlyLeuLysThrValValS
CTGAAGCCGCTATTTCTCTTGGTATGACTACTACCGGGAACACGGCCTGGTTGTCCGCGC
erGluAlaAlaIleSerSerTrpTyrAspTyrTyrArgGluHisGlyLeuValValAlaP
CAAGCGAATGCCAGGGCGAAGACATGGACAAGCTGGCTGAAGTCTGCCAGTCCAACCTCT
roSerGluCysGlnGlyGluAspMetAspLysLeuAlaGluValCysGlnSerAsnLeuT
GGGACGGGGGCAATTTCACTGCTAAAAAGGCTTATGAAGCAGAGCAAGCTGAACTTTTAG
rpAspGlyGlyAsnPheThrAlaLysLysAlaTyrGluAlaGluGlnAlaGluLeuLeuA
CCGCCCAGGACCGGGCAACCGGCCAGTACTCTGATTTCTGGGAGTCCAGAACTACCGCC
laAlaGlnAspArgAlaThrGlyGlnTyrSerAspPheTrpGluSerArgAsnTyrArgH
ACCACACCGATGGCATCAAGTGCTCCTGGATCTCCGTTACGGCTTAAACGACTGGAATG
isHisThrAspGlyIleLysCysSerTrpIleSerValHisGlyLeuAsnAspTrpAsnV
TCAAGCCGAAGAACGTCTACAAGATCTGGCAAAAGGTCAAGCAGCTGCCGGTGAAATCTC
allLysProLysAsnValTyrLysIleTrpGlnLysValLysGlnLeuProValLysSerH
ACCTCTTCCTCCACCAGGGTCCGCACTACAACATGAACAACCTGGTTTCCATCGATTTC
isLeuPheLeuHisGlnGlyProHisTyrAsnMetAsnAsnLeuValSerIleAspPheT
CCGACCTCATGAACCTCTGGTTCGTCCACGAGCTTTTGGAAGTGGAACCGGGGCTTATG
hrAspLeuMetAsnLeuTrpPheValHisGluLeuLeuGluValGluAsnGlyAlaTyrG
AGCAATGGCCAAAGGTCATGATCCAAGACAACCTGGAAGCTGACGAGTGGCACGCAGAAA
luGlnTrpProLysValMetIleGlnAspAsnLeuGluAlaAspGluTrpHisAlaGluS
GTGACTGGGCCAGCGACCTGGGCCAAGCCAGCCTCTATTTACCAACGGCTGACGGGGACC
erAspTrpAlaSerAspLeuGlyGlnAlaSerLeuTyrLeuProThrAlaAspGlyAspL
TGTCACGGTTGAAAACGGGACAGGCCAGCTAACCTTCACCGACTTGGGCGGGACAGAAT
euSerThrValGluAsnGlyThrGlyGlnLeuThrPheThrAspLeuGlyGlyThrGluP
TCAAGAAGGCCGGCATTAGTGAAACTGACTGGGAATACCAGTTTATCAGCGGGGAGGAAA
heLysLysAlaGlyIleSerGluThrAspTrpGluTyrGlnPheIleSerGlyGluGluL
AATGGGCCAAGGCCAGCCTCCGCTTTGAAAGCGAGGAATTCCTCCACCCGACTACCCTGG
ysTrpAlaLysAlaSerLeuArgPheGluSerGluGluPheLeuHisProThrThrLeuV
TCGGCCGGCCAAAGGTCCGGGTCCGGGTAGCAGCTAATAAGACTGTCGGCCAGCTGTCTG
alGlyArgProLysValArgValArgValAlaAlaAsnLysThrValGlyGlnLeuSerV

Figure 6 (cont. 1)

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TTGCCCTGGTTGACCTGGGGACCCGGCAGCGGCTGACGGCTACGCCGAAGATTTTGGCCA
alAlaLeuValAspLeuGlyThrArgGlnArgLeuThrAlaThrProLysIlePheAlaA
GAGGAAACCAGCCCTTTGGCTACCGCTTCGGGGCAGACTCCTTGCAGGAATTCGTCCCGG
rgGlyAsnGlnProPheGlyTyrArgPheGlyAlaAspSerLeuGlnGluPheValProA
ACAAGGCCACTAAGGCCAAGCTGATTACCAAGGCCACATGAACCTGCAGAACTACCAGG
spLysAlaThrLysAlaLysLeuIleThrLysAlaHisMetAsnLeuGlnAsnTyrGlnA
ACATGAAGCAGCCAAGCAAGCTGGAAGCCGGCCAGTTCGTGACCTAGAATTTGAACTGC
spMetLysGlnProSerLysLeuGluAlaGlyGlnPheValAspLeuGluPheGluLeuG
AGCCGACCTACTACACCCTGCCAGCTGGAGCAAAATTGGGTCTGATCATCTATTCAACCG
lnProThrTyrTyrThrLeuProAlaGlyAlaLysLeuGlyLeuIleIleTyrSerThrA
ACCAGGGGATGACCAAGCGGCCGCTGGAGACCGAAGACTACACAGTTGACTTAGCCGGAA
spGlnGlyMetThrLysArgProLeuGluThrGluAspTyrThrValAspLeuAlaGlyT
CTGCTCTTTTGCTCTACCGGAAATAATGCCTGGGCAAAATTGGCGGTTTATGCTAAAT
hrAlaLeuLeuLeuTyrArgLysEnd
AACTTTATCGCAAACAAAGTAAAAGGCTGTGCGGACGTACCGCTGACAGCCTTTTTGTG
TTCTTAGAAAGGTCTTATGGAAAAATCAAGCAAAGTCAACCTTGCTATTTTCAGTGCGG
GCCTGATGACCTTCATCGGTATTCTGAATGAAACCTCCATGAACGTCACCTACCCGCTTT
TAGTCAAAGAGTTCCACCAGCCCCTGGCAACCGTCCAGGTAGATCACTACCGCCTATCTC
TTGACAGTGACCATCGTCATGGGAACCTACCGCCTACCTGCTCAAGCAGGTCCCGGCTCGC
TGGCTGCATTTAGGAGCCACCCTGGCCTTTATTTGCGGTTGCATCATCTGCGCCTTAACT
TCCAGCTTTCCTTTGATGCTGGCCGGCCGGATCATTCAGGGGATCGCCACCGGCTTTTCC
ACCCCGATCATGTTCCAGCTGATCTTTACCCAGGTCCCTAAGCAAAAACCTCGGCCTCATG
ACCGGTTTTGCGGGGACGATCATCTCCTTTGCCCC

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	10	20	30	40	50	60
11bpepx	.	.	.	.	.	.
111xpdap	MKYNQAYVETSP	EATELLA	INFLPEN	YSSLSF	SELLAVL	TGNVLA
111pepxp	MRFNHFSIVDK	NFDEQLA	ELDQLGF	-----	RWSVF	WDEKKIL
	MRFNHFSIVDK	NFDEQLA	ELDQLGF	-----	RWSVF	WDEKKIL
	*	*	*	*	*	*
11bpepx	AEFAVDDQTD	LAAFLD	TPTAIT	ASQFAN	VALQLL	GYHPNY
111xpdap	TDLQATAELD	VIEFL	-KSSIE	LDEWEIF	WNIALQ	LLDFVP
111pepxp	TALQATAELD	VIEFL	-KSSIE	LDEWEIF	WNIALQ	LLDFVP
	*	*	*	*	*	*
11bpepx	KDLTSKEELI	FTFYRL	LNTRSK	NGQILL	DVMAGK	GYFTQF
111xpdap	EAEMTTENII	ISAFYLL	CTRRKN	GMI	VEHWV	SEG
111pepxp	EAEMTTENII	ISAFYLL	CTRRKN	GMI	VEHWV	SEG
	*	*	*	*	*	*
11bpepx	QVIREVVYQ	SDLD	TDGDGK	GDLLP	VTVFR	PVESQD
111xpdap	LLEREVLW	ESPVD	SEQR	ENDLI	KIQIIR	PKSTE
111pepxp	LLEREVLW	ESPVD	SEQR	ENDLI	KIQIIR	PKSTE
	***	*	*	*	*	*
11bpepx	TNHNVDENL	TDAT	TWTNPK	YVAKPL	VKSPAP	SDQDVP
111xpdap	ALHDMNVE	LEEK	T--SHEI	HVEQK	PLQKLSA	KAKELP
111pepxp	ALHDMNVE	LEEK	T--SHEI	HVEQK	PLQKLSA	KAKELP
	*	*	*	*	*	*

Figure 7

SUBSTITUTE SHEET

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1bpepx	RGFASVFGAIGNRHGDIRITGSPEETISQKEVIEWLTDGRVAYTDTRRRFETKASWCS
11xpdap	RGFASIYVAGVGTSSDGFQTSQDYQQIYSMTAVIDWLNCRARAYTSRKKTHEIKASWAN
11pepxp	RGFASIYVAGVGTSSDGFQTSQDYQQIYSMTAVIDWLNCRARAYTSRKKTHEIKASWAN
	***** *
1bpepx	GNVGMTGRSYLGLTLQIAIATTGVKGLKTVVSEAAISSWYDYREHGLVAPSECQGEDMD
11xpdap	GKVAMTGKSYLGTMYGAATTGVEGLEVILAEAGISSWYNYRENGLVRSPPGGFPGEDLD
11pepxp	GKVAMTGKSYLGTMYGAATTGVEGLELILAEAGISSWYNYRENGLVRSPPGGFPGEDLD
	* * * * * ***** ** * * * * * * * * * * * * * * *
1bpepx	KLAEVCQSNLWDGGNF-TAKKAYEAEQAEELAAQDRATQXSDFWESRNYRHHTDGIKCS
11xpdap	VLAALTYSRNLDGADFLKGNAEYKRLAEMTAALDRKSGDYNQFVHNRNYLLINTDKVKAD
11pepxp	VLAALTYSRNLDGADFLKGNAEYKRLAEMTAALDRKSGDYNQFVHNRNYLLINTDKVKAD
	** * * * * ** * * * * * * * * * * * * * * * * * *
1bpepx	WISVHGLNDWNVPKNVYKIWKVKQLPVKSHLFLHQPHYNNMNLVSIDFTDLMLNLFV
11xpdap	VLI VHGLQDWNVTPEQAYNFWKALPEGHAK-HAFLHRGAHIYMNSWQSIDFSETINAYFV
11pepxp	VLI VHGLQDWNVTPEQAYNFWKALPEGHAK-HAFLHRGAHIYMNSWQSIDFSETINAYFV
	***** *
1bpepx	HELLEVENGAYEQWPKVMIQDNLEADWEHAEESDASDLGQASLYLPTADGDLSTVENGTG
11xpdap	AKLLDRDLNL--NLPPVILQENSKDQVWTMMNDFGAN-TQIKLPLGKTAVSFAQFDNHYD
11pepxp	AKLLDRDLNL--NLPPVILQENSKDQVWTMMNDFGAN-TQIKLPLGKTAVSFAQFDNHYD
	** *
1bpepx	QLTFTDLGCT--EFKKAGISETDWEYQFISGEEKWAKASLRFESSEFLHPTTLVGRPKVR
11xpdap	DETFKKYSKDFNVFKKD-----LF---ENKANEAVIDLELPSML---TINGPVELE
11pepxp	DETFKKYSKDFNVFKKD-----LF---ENKANEAVIDLELPSML---TINGPVELE
	** *

Figure 7 (cont. 1)

SUBSTITUTE SHEET

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1bpepx VRVAANKTVGQLSVALVDLGTRQRLTATPKIFARGNQPFYRFGADSLQEFVDPDKATKAK
 11xpdap LRLKLNDTKGFLSAQILDGQKKRLEDKARVKDFKVLDRGRNFMDDDLVEL-PLVESPYQ
 11pepxp LRLKLNDTKGFLSAQILDGQKKRLEDKVRVKDFKVLDRGRNFMDDDLVEL-PLVESPYQ
 * * * * * * * * * * * * * * *

1bpepx LITKAHMNLQNYQDMKQPSKLEAGQFVDLEFELQPTYTYTLPAGAKGLIITYSTDQGMTRK
 11xpdap LITKGFTNLQN-QNLLTVSDLKADEWFTTIKFELQPTIYHLEKADKLRVILYSTDFEHTVR
 11pepxp LVTKGFTNLQN-QSLLTVSDLKADEWFTTIKFELQPTIYHLEKADKLRVILYSTDFEHTVR
 * * * * * * * * * * * * * * *

1bpepx PLETEDYTVDLAGTALL----YRK
 11xpdap DNRKVTYEIDLSQSKLIPIESVKN
 11pepxp DNRKVTYEIDLSQSKLIPIESVKN
 * * * *

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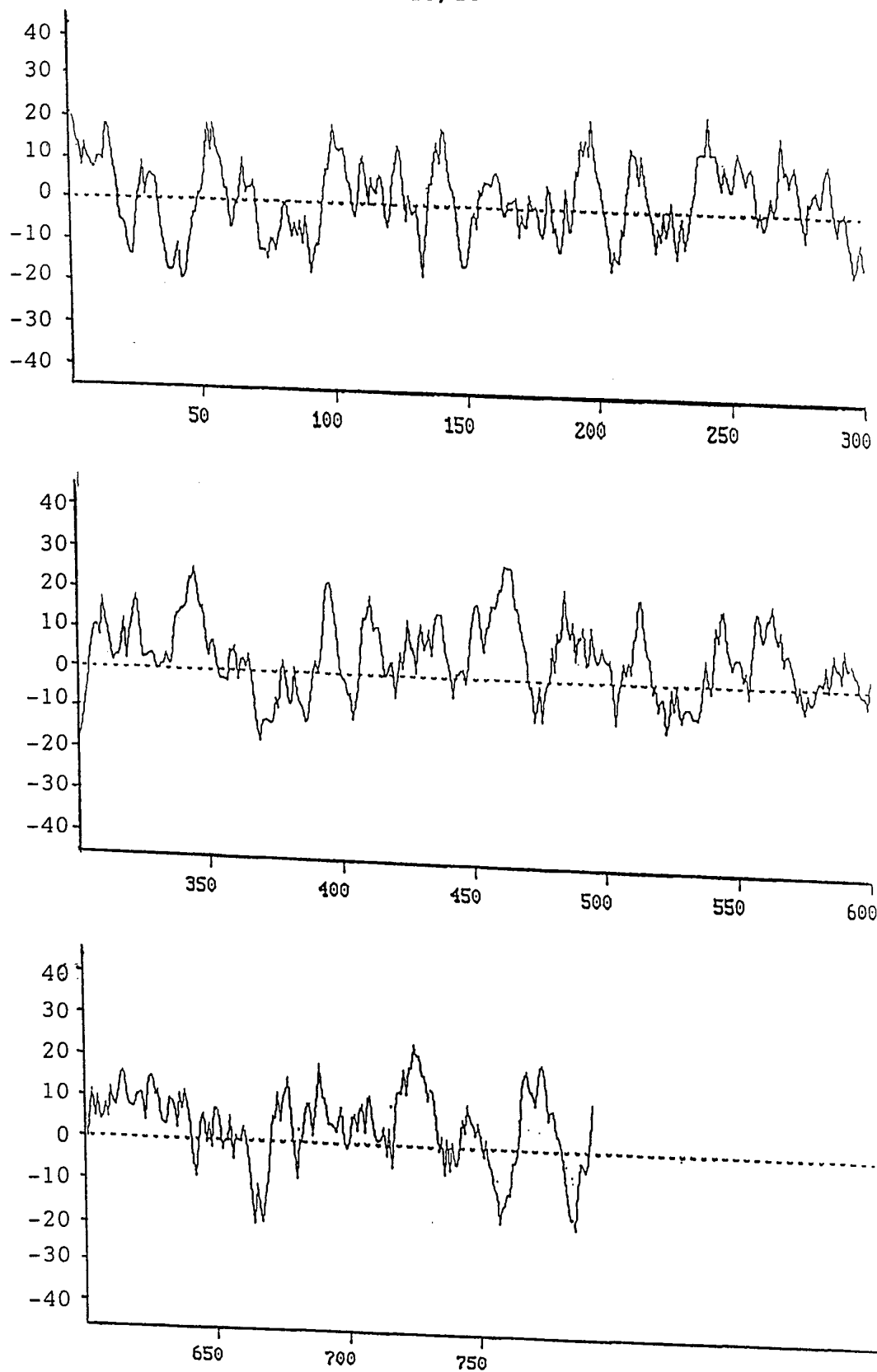


Figure 8
SUBSTITUTE SHEET

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

24 JAN 1994

Prof. Dr. R. Plapp
Abt. Mikrobiologie
der Universität
Kaiserslautern
Erwin-Schrödinger-Str. 23
Postfach 3049
6750 Kaiserslautern

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Prof. Dr. R. Plapp Abt. Mikrobiologie Address: der Universität Kaiserslautern Erwin-Schrödinger-Str. 23 Postfach 3049 6750 Kaiserslautern	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 7290 Date of the deposit or of the transfer ¹ : 1992-10-13
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 1992-10-13 ² On that date, the said microorganism was (X) ³ viable () ³ no longer viable	
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED ⁴	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSM DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Maacheroder Weg 1 B D-3300 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 1992-10-16

¹ Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

³ Mark with a cross the applicable box.

⁴ Fill in if the information has been requested and if the results of the test were negative.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 93/03711

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/57 C12N15/74 C12N9/52 A23C19/032

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 5 C12N A23C

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

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	INT. DAIRY JOURNAL vol. 1, 1991 pages 51 - 66 WILHEM BOCKELMANN ET AL. 'Purification and characterization of the X-prolyl-dipeptidyl-aminopeptidase from Lactobacillus delbrueckii subsp. bulgaricus and Lactobacillus acidophilus' see abstract see page 51, paragraph 1 - page 52, paragraph 3 see page 54, paragraph 3 - page 55, paragraph 3 see page 55, paragraph 5 - page 62, paragraph 1 see page 62, paragraph 3 - page 63, paragraph 2; tables 2,3 --- -/--	1-5, 15

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in 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 but 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.

"&" document member of the same patent family

Date of the actual completion of the international search

29 April 1994

Date of mailing of the international search report

15 -06- 1994

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 Fax: (+31-70) 340-3016

Authorized officer

Montero Lopez, B

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 93/03711

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF DAIRY SCIENCE vol. 70, no. 4 , April 1987 , CHAPAIN, ILLINOIS US pages 738 - 745 JACQUES MEYER ET AL. 'Purification and characterization of X-prolyl-dipeptidyl-aminopeptidases from Lactobacillus lactis and from Streptococcus thermophilus' see page 738, left column, paragraph 1 see page 738, right column, paragraph 6 - page 739, right column, paragraph 2 see page 739, right column, paragraph 4 - page 740, left column, paragraph 1 see page 740, right column, paragraph 6 - page 741, right column, paragraph 1; table 2 see page 744, left column, paragraph 2 - page 745, left column, paragraph 5 ---	1-5,15
A	JOURNAL OF DAIRY SCIENCE vol. 74, no. 8 , August 1991 , CHAPAIN, ILLINOIS US pages 2375 - 2381 HIROSHI MIYAKAWA ET AL. 'Purification and characterization of an X-prolyl dipeptidyl aminopeptidase from Lactobacillus delbrueckii ssp. bulgaricus LBU-147' cited in the application see page 2375, left column, paragraph 1 see page 2375, right column, paragraph 4 see page 2377, left column, paragraph 2 - right column, paragraph 2 see page 2378, right column, paragraph 1 see page 2379, left column, paragraph 3 - page 2381, left column, paragraph 3 ---	1-5, 15-19
P,X	APPLIED MICROBIOLOGY AND BIOTECHNOLOGY vol. 40, no. 1 , October 1993 pages 82 - 89 ELKE CHRISTEL MEYER-BARTON ET AL. 'Cloning and sequence analysis of the X-prolyl-dipeptidyl-aminopeptidase gene (pepX) from Lactobacillus delbrückii ssp. lactis DSM7290' see abstract see page 82, right column, paragraph 4 see page 83, left column, paragraph 5 see page 83, right column, last paragraph - page 84, right column, paragraph 2; figure 4 ---	1-3, 5-10, 12-15
2		
3	P,X EMBL Database entry Accession number S32244; 22 November 1993 MEYER, E. ET AL. ---	1,3
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 93/03711

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EMBL Database entry Accession number Z14230; 31 March 1993 MEYER E. ET AL.: 'Purification, characterization and sequence analysis of X-prolyl-dipeptidyl- aminopeptidase from <i>Lactobacillus</i> <i>delbrueckii</i> ssp. <i>lactis</i> WS87' -----	6