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Purification and characterization of a proline iminopeptidase from *Propionibacterium shermanii* 13673

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Summary — A proline iminopeptidase activity was found in *P shermanii* 13673. The enzyme activity was present in the intracellular fraction. The peptidase was purified approximately 80 times by ion-exchange chromatography on Fractogel TSK DEAE 650 and gel filtration on Sephacryl S-100 HR. The purified enzyme appeared as a single band after sodium dodecyl sulfate–polyacrylamide gel electrophoresis. It had a molecular weight of 61 000. Optima for activity of the purified enzyme were 40 °C and pH 8.0. The enzymatic activity was inhibited by phenylmethylsulfonylfluoride (1 mM). Divalent ions Zn, Co, Cu and Fe also had an inhibitory effect. The peptidase hydrolyzed specifically Pro *p*-NA amino acid, dipeptides Pro-Met, Pro-Phe, Pro-Le, Pro-Ileu, Pro-Gly and β-casomorphin des-Tyr fragment 7.

***Propionibacterium shermanii* / enzyme / peptidase / proline / purification**

Résumé — Purification et caractérisation d'une proline iminopeptidase à partir de la souche *Propionibacterium shermanii* 13673. *Une activité de proline iminopeptidase a été mise en évidence dans la souche* P shermanii *13673. L'activité enzymatique est présente dans la fraction intracellulaire. L'enzyme a été purifiée environ 80 fois par chromatographie d'échange d'ions sur fractogel TSK DEAE 650 et gel filtration sur Sephacryl S-100 HR. L'enzyme purifiée présente une seule bande après électrophorèse en gel de polyacrylamide et en présence de SDS. Elle a un poids moléculaire de 61 000. La température est de 40°C et le pH optimal de 8,0. L'activité enzymatique est inhibée par le phénylméthylfulfonyl fluoride (1 mM). Les ions divalents Zn, Co, Cu et Fe ont aussi un effet inhibiteur. La peptidase hydrolyse spécifiquement la Pro* p-*NA, les dipeptides Pro-Met, Pro-Phe, Pro-Leu, Pro-Ile, Pro-Gly et le fragment 7 des-Tyr de la β-casomorphine.*

Propionibacterium shermanii / *enzyme / peptidase / proline / purification*

INTRODUCTION

Proteolysis is probably the most important biochemical event during the ripening of most cheese varieties with a major impact on flavor and texture. The mechanisms of flavor compound formation in hard cheeses involve successive steps and several agents: rennet, indigenous milk proteinases, starter proteinases and peptidases, enzymes from secondary starters and from non starter microorganisms. Thermophilic acid bacteria such as *Streptococcus thermophilus*, *Lactobacillus helveticus* and *Lactobacillus lactis* are normally used as starter bacteria in Swiss cheese industries, whereas propionic acid bacteria are added as secondary starters. The initial lactic fermentation by the starters is followed by fermention of lactate and residual sugars due to presence of propionic acid bacteria. This is a vital stage in Swiss cheese ripening.

Enzyme activities and proteolysis during cheese maturation have been reviewed by Fox (1989) and by Kamaly and Marth (1989). Purification of peptidases from starter bacteria has been reported by Rabier and Desmazeaud (1973), Desmazeaud and Juge (1976), Eggimann and Bachmann (1980), Desmazeaud (1982), El Soda and Demazeaud (1982), Ezzat *et al* (1982), Hickey *et al* (1983), Casey and Meyer (1985), Meyer and Jordi (1987) and Khalid and Marth (1990).

Proteolytic system from secondary starters has also been studied by Langsrud (1974). Four or 5 peptidases in a strain of *Propionibacterium shermanii* have been detected, one of which was a proline iminopeptidase. Langsrud *et al* (1977) found that propionibacteria released large amounts of proline when grown in media containing peptides. Langsrud *et al* (1978b) established that propionibacteria also produced intracellular peptidases which were released by autolysis in hard cheeses. Sahlstrom *et al* (1989) found peptidase activities in cell wall, membrane and intracellular fractions of *P shermanii*. Perez Chaia *et al* (1990), studying the activity of peptidases belonging to propionibacteria, found a greater affinity for proline *p*-nitroanilide than for leucine *p*-nitroanilide.

In this work we have purified (with good reasons for presuming homogeneity) and characterized a proline iminopeptidase from *P shermanii* 13673. This is the first report describing the purification and characterization of a proline iminopeptidase from a cell extract of propionic acid bacteria.

MATERIAL AND METHODS

Chemicals

Lysozyme, mutanolysin, DNase, RNase, all peptide derivatives were obtained from Sigma Chemical Co, St Louis, MO, USA. Fractogel TSK DEAE 650 was purchased from E Merck, Darmstadt, Germany. Sephacryl S100 HR and molecular weight markers were obtained from Pharmacia-LKB, Saint-Quentin en Yvelines, France.

Bacterial strains

Five strains from our collection were studied. The strain of *P globosum* 408 was supplied by a starter bacteria producer, Standa-Industrie, Caen, France. The strain of *P freudenreichii* CNRZ 435 was supplied from the culture collection of the Centre de Recherches (CNRZ), Jouy-en-Josas, France. *P shermanii* 13673 and the 2 strains of *P acidi propionici* 4875 and P01 were obtained from one private Emmental industry.

All strains were identified according to *Bergey's Manual of Determinative Bacteriology* (More and Holdeman, 1974) and the method of Britz and Steyn (1980) and the method of Britz

and Steyn (1980) and also by control of propionic acid production (HPLC method).

Growth conditions

The strains were maintained on yeast extract lactate (YEL) medium (Hettinga *et al*, 1968): sodium lactate 1% (Merck), K_2HPO_4 0.025% (Prolabo), $MgSO_4$ 0.005% (Prolabo), biotrypticase 1% (BioMérieux), yeast extract 1% (Biokar); pH was ajusted to 7.0 and medium autoclaved for 15 min at 121°C. Strains were transferred 3 times in 10-ml tubes. Cultures of 2 l were grown in YEL medium with an inoculum of 1%. Growth was maintained at 30°C for 48 h.

Preparation of cell extracts

At the end of the exponential growth period the cells were harvested and centrifuged at 10 000 *g* for 15 min. The supernatants were stored at –20 °C. The cell pellets were washed twice in 200 ml of 50 mM β glycerophosphate, pH 7.0.

Washed cells were then treated by protoplast buffer: 100 ml of 50 mM/l Tris buffer, pH 7.0 containing 0.5 M saccharose, lysozyme at 5 mg/ml and mutanolysin at 5 µg/ml (Kondo and McKay, 1982). After incubation at 37°C for 2 h the protoplasts were recovered by centrifugation at 5 500 *g* for 20 min. Protoplast supernatants were stored at –20 °C after filtration. Cells were disrupted by osmotic shock in alkaline buffer (pH 12). pH was immediately adjusted to 7.0 with 2 M Tris buffer.

After DNase and RNase treatment (0.1 mg/ml) the crude extract was then centrifuged at 24 000 *g* for 30 min at 4°C in order to eliminate intact cells and cell debris. The cell-free extracts designated as the cytoplasmic or intracellular fractions were stored at –20°C, before aminopeptidase activity investigations. The intracellular fraction of *P shermanii* 13673 was used as starting material for the purification of the peptidase.

Substrate specificity

Intracellular fractions were tested with L-leucine *p*-nitroanilide (Leu *p*-NA), L-lysine *p*-nitroanilide (Lys *p*-NA), L-Proline *p*-nitroanilide (Pro *p*-NA) and L-glycine *p*-nitroanilide (Gly *p*-NA) substrates. Protein extracts from purification were routinely tested with Pro *p*-NA substrate.

Aminopeptidase activity was assayed by incubation of intracellular preparation with 0.75 mM *p*-nitroanilide amino acids in 20 mM Tris–HCl buffer pH 7.0. Incubation was carried out at 37°C for 1 h. The reaction was stopped by the addition of 30% acetic acid. The supernatant was separated by centrifugation at 10 000 *g* for 5 min. The release of *p*-nitroanilide was followed by measuring absorbance at 410 nm (A_{410}) in an Uvikon Kontron 860 spectrophotometer. One unit of aminopeptidase activity was defined as the amount of enzyme which produced a variation of 0.01 unit of A_{410} per min at 37 °C. The purified enzyme was also tested against several di- and tripeptides. Purified enzyme was incubated with 0.4 mM substrate in phosphate buffer pH 7.0. After incubation at 37°C for 1 h, free α amino groups were determined with 1 vol of ninhydrin (20 g of ninhydrin, 750 ml of methylcellosolve (ethylene glycol monomethyl ether), 250 ml of 4 M acetate buffer (pH 5.51), 7 ml of titanium chloride). The preparation was boiled at 100°C for 5 min and 3 vol of 50% ethanol were added. Hydrolysis was followed at A_{440} for proline, and A_{570} for the other amino acids.

Substrate specificity of the peptidase was also tested by HPLC against bovine β-casomorphin fragments: fragment 1–3, morphoceptin (β-casomorphin 1–4 amide), fragment 1–5 and des-Tyr-fragment 7. The purified enzyme was incubated with 0.4 mM substrate in 20 mM Tris buffer pH 7.0 for 3 h at 40°C. The reaction mixtures and substrates were injected onto a Vydac column C 18 5 µ. Peptides were eluted by a gradient water/acetonitrile (1 vol/4 vol).

Purification

First ion exchange chromatography

The first ion exchange chromatography was performed with a Fractogel TSK DEAE 650 column. The column (2.6 cm x 40) was equilibrated with 20 mM Tris–HCl buffer pH 7.0. One hundred ml of intracellular fraction of *P shermanii* 13673 were dialyzed and centrifuged. This fraction (3 000 mg) was applied to the column. After

washing the column with the equilibration buffer, the enzyme was eluted with a linear gradient of 0 to 0.6 M NaCl in the same buffer at a flow rate of 90 ml/h. Fractions of 11.2 ml each were collected and tested for protein concentration by measuring absorbance at 280 nm (A_{280}). Peptidase activity was tested with Pro *p*-NA substrate. Active fractions (Nos 36–45) were pooled and stored at –20 °C in the presence of stabilizers according to the method of Neviani *et al* (1989).

Second ion exchange chromatography

The enzyme active fractions of the first performed chromatography were dialyzed against 20 mM Tris–HCl pH 7.0 at 4 °C and applied to the same Fractogel TSK DEAE column previously equilibrated with 20 mM Tris–HCl buffer pH 7.0. The column was washed with 20 mM Tris–HCl; the enzyme was eluted with a linear gradient of NaCl 0–0.5 M in 20 mM Tris–HCl pH 7.0 at a flow rate of 90 ml/h, and 11.2-ml fractions collected. Active fractions (Nos 38–46) against Pro *p*-NA substrate were pooled and dialyzed against 20 mM Tris–HCl buffer pH 7.0 at 4°C. They were concentrated by PEG 3000 before gel filtration.

Gel filtration

The concentrated enzyme (9 ml) was further purified on a Sephacryl S-100 HR column (2.6 cm x 100) previously equilibrated with 20 mM Tris–HCl buffer pH 7.0 containing 1 M NaCl. A flow rate of 120 ml/h was applied and 8-ml fractions were collected from the column. The enzyme was eluted from the column as a single peak. Active fractions (Nos 11–15) against Pro *p*-NA substrate were pooled and dialysed against 20 mM Tris–HCl buffer pH 7.0 at 4 °C. Characterization of the enzyme was performed with this preparation.

Polyacrylamide gel electrophoresis

Each purification step was examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with the Laemmli buffer system (Laemmli, 1970). A 3% stacking gel and a 10% running gel were prepared. The protein samples were mixed 1/1 with sample buffer (10 mM Tris–HCl pH 6.8, 20% glycerol, 2% SDS, 5% 2-mercapto-ethanol and 0.001% bromophenol blue), boiled for 5 min, and applied to the gel. After a 6-h run at 150 V, proteins were stained with Coomassie blue R. For destaining, the gels were put into a solution of 10% (v/v) acetic acid, 10% (v/v) methanol in water.

Determination of molecular weight

Molecular size of the active enzyme was estimated by gel filtration on Sephacryl S-100 HR in column 2.6 x 100. The column was first calibrated with bovine serum albumin (67 kDa), ovalbumin (43 kDa) and lysozyme (14.3 kDa). Molecular size was also estimated by SDS–PAGE. The low molecular weight standards used were obtained from Pharmacia (Uppsala, Sweden): phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), bovine carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). The molecular weight of the aminopeptidase was determined by a reference to a standard graph relating log of molecular weight of each standard to its relative mobility.

Effect of reducing agents, metal chelators and inhibitors on enzyme activity

A diverse range of compounds known to inhibit various proteases were tested for their effects on the peptidase activity of *P shermanii* 13673. Reducing agents and thiol, metallo, serine protease inhibitors were tested. The enzyme was preincubated in 20 mM Tris–HCl pH 7.0 for 15 min at 20 °C with the following agents: dithiothreitol, L-cysteine, 2-merceptoethanol, *N*-ethylmaleimide, iodoacetic acid, iodoacetamide, *p*-chloromercuric benzoic acid, ethylenediaminetetraacetic acid (EDTA), 1-10 phenanthroline, bestatin, phosphoramidon, phenylmethylsulfonyl fluoride, pepstatin A and sodium citrate. Two final concentrations were used: 0.1 mM and 1 mM (1 and 10 mM for EDTA). Pro *p*-NA was added as substrate and enzyme activity was measured after incubation at 37 °C for 60 min at A_{410}.

Effect of metal ions on enzyme activity

Effects of divalent ions were tested on enzyme activity. The purified and dialyzed enzyme was incubated in the presence or the absence of Ca chloride, Co acetate, Cu sulfate, Zn, Mn sulfate and Mg chloride at a final concentration of 0.1 mM. Reaction mixtures were preincubated for 15 min at 20 °C in 20 mM Tris–HCl buffer (pH 7.0) prior to the addition of Pro *p*-NA. The enzyme activity was determined at 37°C for 60 min at A_{410}.

Protein estimation

The BioRad method (BioRad Laboratories, Watford, England) was used with bovine serum albumin as standard (Bradford, 1976). Elution profiles of the proteins separated after Fractogel TSK DEAE 650 and Sephacryl S-100 HR were determined at A_{280}.

Effect of pH on enzyme activity

The effect of pH from 4.0 to 10.0 on peptidase activity was determined by using 20 mM glycine–NaCl or Tris–HCl buffer adjusted to the appropriate pH values. Enzyme activity was measured at 37°C for 60 min with Pro *p*-NA as substrate at A_{410}.

Effect of temperature on enzyme activity

The effect of temperature was measured in the range of 5–70°C. The 20 mM Tris–HCl buffer (pH 7.5) was equilibrated for 15 min at the test temperature. Fifty µl of purified enzyme and 50 µl of Pro *p*-NA as substrate were added and the enzyme activity was measured after 60 min of incubation at the test temperatures at A_{410}.

Amino acid analysis

The purified enzyme was hydrolyzed with 6 N HCl under vacuum at 110 °C for 24 h. Amino acids were quantitatively assayed with an amino acid analyzer Pharmacia-LKB alpha plus.

RESULTS

Peptidase purification

Enzymatic treatment of propionibacterial cells was preferred to physical treatment because it does not generate high shear stresses and minimises product damage (Andrews and Asenjo, 1987). Peptidase activity was observed in the cell wall fraction (data not shown), probably due to partial cell lysis during protoplast formation.

The major peptidase activities with leucine, lysine, proline and glycine *p*-nitroanilide substrates were present in the soluble intracellular fraction of 5 strains of propionibacteria (table I). For all extracts

Table I. Hydrolysis of *p*-nitroanilide (*p*-NA) substituted amino acids (leucine, lysine, proline, glycine) by intracellular fraction of *P globosum* 408, *P freudenreichii* CNRZ 435, *P shermanii* 13673, *P acidi propionici* 4875, *P acidi propionici* PO1. Results are expressed in units of activity per min at 37°C.
Hydrolyse des acides aminés (leucine, lysine, proline, glycine) substitués (p-NA) par les fractions intracellulaires de P globosum *408,* P freudenreichii *CNRZ 435,* P shermanii *13673,* P acidi propionici *4875,* P acidi propionici *PO1. Les résultats sont exprimés en unités d'activité par min à 37 °C.*

	Substrate			
	Leu p-*NA*	*Lys* p-*NA*	*Pro* p-*NA*	*Gly* p-*NA*
P globosum 408	75	0	180	75
P freudenreichii Z 435	150	0	750	0
P shermanii 13673	180	150	2 660	0
P acidi propionici 4875	100	0	500	0
P acidi propionici PO1	200	0	600	0

we observed a stronger activity with Pro *p*-NA than with Leu *p*-NA substrate.

After this result we decided to study *P shermanii* 13673 more particularly and to purify the specific aminopeptidase. In the first ion exchange chromatography performed on Fractogel TSK DEAE 650 the proline activity was eluted from the column at 0.4 M NaCl (fig 1). During the second ion exchange chromatography on the same Fractogel TSK DEAE 650, the enzyme was eluted at 0.4 M NaCl.

After gel filtration on Sephacryl S-100 HR the enzyme was eluted from the column as a single and symmetrical peak (fig 2). Results of the purification are summarized in table II. Using this protocol the proline iminopeptidase was purified ≈ 80-fold with a yield of 7% from the crude extract.

Peptidase characterization

Molecular weight determination

The enzyme preparation showed a single band after SDS–PAGE. The molecular weight of the enzyme was estimated at 61 000 even in the presence of 2-mercaptoethanol (data not shown) (fig 3). The peptidase was also found to have a molecular weight of 61 000 by gel filtration.

Effect of pH and temperature on enzyme activity

The optimal pH for Pro *p*-NA hydrolysing activity appeared to be pH 8.0 (fig 4A). At pH 5.0 and 9.0, hydrolysing activity was also detected. The optimal temperature for

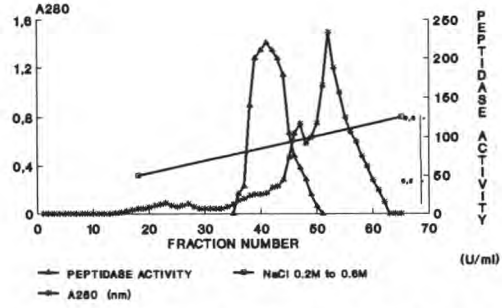

Fig 1. First ion exchange chromatography. Purification of aminopeptidase from intracellular fraction of *P shermanii* 13673. Elution pattern of the peptidase from a Fractogel TSK DEAE 650 column. The peptidase activity λ (A_{410}) was assayed with Pro *p*-NA substrate.
Première chromatographie d'échange d'ions. Purification de l'aminopeptidase à partir de la fraction intracellulaire de P shermanii *13673. Profil d'élution de la peptidase de la colonne de Fractogel TSK DEAE 650. L'activité de la peptidase λ (A_{410}) a été testée avec le substrat Pro* p-*NA.*

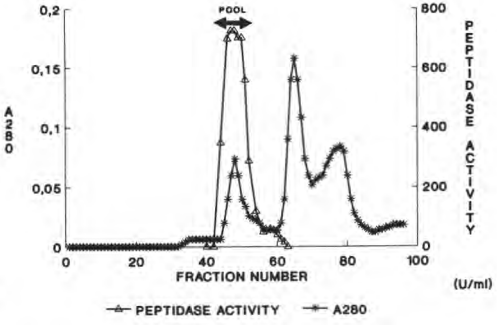

Fig 2. Gel filtration on Sephacryl S-100 HR. Purification of peptidase from *P shermanii* 13673 after the second ion exchange chromatography on a Sephacryl S-100 HR column. The peptidase activity λ (A_{410}) was assayed with Pro *p*-NA substrate.
Gel filtration sur Sephacryl S-100 HR. Purification de la peptidase de P shermanii *13673 après la seconde chromatographie d'échange d'ions sur colonne de Sephacryl S-100 HR. L'activité de la peptidase λ (A_{410}) a été testée avec le substrat Pro* p-*NA.*

Table II. Purification of the peptidase from *P shermanii* 13673.
Purification de la peptidase de P shermanii *13673*

Purification step	*Vol (ml)*	*Total protein (mg)*	*Total* activity (U)*	*Specific activity (U/mg)*	*Yield (%)*	*Purification (fold)*
Crude cell extract	100	3 000	14 248	4.75	100	1
1st Fractogel TSK DEAE 650	100	110	2 330	21.2	16.3	4.4
2nd Fractogel TSK DEAE 650	100	40	2 260	56.5	15.8	11.9
Sephacryl S-100 HR	10	2.7	992	367	7	77.2

* Determined with the substrate Pro *p*-NA; * *Déterminé avec le substrat Pro* p-*NA*

Pro *p*-NA hydrolysing activity was found to be 40 °C (fig 4B). Significant activity was detected at 35 and 45°C; at 20°C, 50% of specific activity was detected, but at 55°C only 9% of the optimal activity was noted.

Substrate specificity

The purified enzyme was incubated with *p*-nitroanilide derivatives of amino acids or dipeptides, and with dipeptides and tripeptides (table III). Pro *p*-NA had the highest activity among the *p*-NA substrates. The purified enzyme catalyzed hydrolysis of dipeptides only if they had a proline residue in the N-terminal position: Pro-Phe, Pro-Met, Pro-Leu, Pro-Ile, Pro-Gly. Carbobenzoxy peptides were not hydrolyzed by the enzyme. The purified peptidase had the same high specificity for Pro *p*-NA as the intracellular fraction. The peptidase had no action on β-casomorphin fragments 1–3, 1–4 and 1–5 (table IV). But the peptidase was able to hydrolyze specifically the β-casomorphin des-Tyr-fragment 7: Pro-Phe-Pro-Gly-Pro-Ile. After 3 h of incubation at 40°C the peptidase hydrolysed ≈ 50% of the substrate. This specific hydrolysis was continuous with time (data not shown). For identification of released products we injected proline as a marker. Under our conditions; Pro was not detectable. It is likely that the peak which appeared was the Phe-Pro-Gly-Pro-Ile peptide. Morever, this could be confirmed by identification of this peptide by amino acid analysis.

Effect of chemical reagents and inhibitors

Treatment of the peptidase with several agents is summarized in table V. The rate of Pro *p*-NA hydrolysis in the absence of any reducing agent, metal chelator or inhibitor was taken as 100%. The enzyme activity was reduced to 30% when incubated in 1.0 mM phenylmethylsulfonyl fluoride (PMSF), a specific inhibitor for serine protease. The metal-complexing agents EDTA (10 mM) and bestatin also decreased enzyme activity, indicating a metal ion requirement. Sulfhydryl inhibitors had some effect on the proline iminopeptidase activity, whereas dithiothreitol and 2-

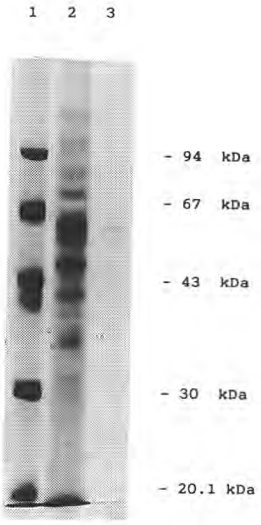

Fig 3. SDS–polyacrylamide gel electrophoresis. 1, Low molecular weight standards: from top to bottom, phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), bovine carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa). 2, Crude intracellular extract from *P shermanii* 13673. 3, Purified peptidase (1.6 μg of protein) after gel filtration on Sephacryl S-100 HR.
Electrophorèse en gel de SDS–polyacrylamide. 1, Standards de faible poids moléculaire : de haut en bas, phosphorylase b (94 kDa), albumine bovine (67 kDa), ovalbumine (43 kDa), anhydrase carbonique bovine (30 kDa), inhibiteur trypsique de soja (20.1 kDa); 2, Extrait intracellulaire brut de P shermanii *13673; 3, Peptidase purifiée (1.6 μg de protéine) après gel filtration sur Sephacryl S-100 HR.*

mercaptoethanol had no effect on enzyme activity.

Several divalent metal ions were tested (fig 5). Addition of 0.1 mM Co acetate, Cu sulfate, Fe sulfate inhibited the enzyme activity by 20, 53 and 77% respectively. A complete inhibition was observed with 0.1 mM Zn. Addition of 0.1 mM Ca and Mg chloride had no inhibitory effect.

Amino acid composition

The amino acid composition is shown in table VI. The peptidase contained moderate quantities of hydrophobic amino acids, consistent with the intracellular origin of the protein.

DISCUSSION

In the present study a proline iminopeptidase was purified from the intracellular fraction of *P shermanii* 13673 in several steps. Purification, as estimated from the specific activity, was approximately 80-fold with a yield of 7%; but protein purification of ≈ 1 000-fold was achieved. During the different steps of purification, inactivation apparently occurred in spite of the presence of stabilizers. The proline iminopeptidase of *P shermanii* 13673 has a molecular weight of 61 000 as estimated by SDS–PAGE and gel filtration. In comparison the aminopeptidase from *S thermophilus* has a molecular weight of 62 000, and its dipeptidase 50 000 (Rabier and Desmazeaud, 1973), whereas the aminopeptidase from *L lactis* has a molecular weight of 78 000 to 81 000 (Eggimann and Bachmann, 1980). The prolyl-dipeptidyl aminopeptidase from *L helveticus* has a molecular weight of 72 000 (Khalid and Marth, 1990) and the X-prolyl-dipeptidyl-aminopeptidase from *L lactis* and from *S thermophilus* has a molecular weight of ≈ 165 000 Da (Meyer and Jordi, 1987). Incubation of the enzyme with dithiothreitol or 2-mercaptoethanol had no effect on enzyme activity, suggesting that an intact disulphide group was not essential for the mechanism of action of this enzyme. The enzyme was more markedly inhibited by phenylmethylsulphonyl fluoride (1 mM). These results suggest that the enzyme is a serine protease. Bacterial dipeptidyl aminopeptidases from *L lactis*, and *S*

Table III. Relative activity* of the peptidase with different substrates.
Activité relative de la peptidase avec différents susbstrats.*

Substrate	*Relative activity (%)*	*Substrate*	*Relative activity (%)*
Pro *p*-NA	100	Pro-Phe	100
Leu *p*-NA	0	Pro-Met	75
Lys *p*-NA	0	Pro-Leu	50
Gly *p*-NA	0	Pro-Ile	40
Met *p*-NA	0	Pro-Gly	16
Glu *p*-NA	0	Phe-Pro	0
Phe *p*-NA	0	Met-Pro	0
Gly-Pro *p*-NA	0	Val-Pro	0
Arg-Pro *p*-NA	0	Lys-Phe	0
Gly-Arg *p*-NA	0	Met-Ala	0
Phe-Val *p*-NA	0	Leu-Ala	0
		Met-Asp	0
		Phe-Phe	0
		Leu-Leu	0
		Ala-Phe	0
		Tyr-Ala	0
		Leu-Phe	0
		Ala-Leu	0
		Leu-Tyr	0
		Leu-Pro	0
		His-Phe	0
		Met-Leu	0
		Phe-Tyr	0
		Ala-Pro	0
		Leu-Gly	0
		Gly-Phe	0
		Leu-Gly-Gly	0
		Ala-Leu-Gly	0
		Leu-Leu-Leu	0
		Phe-Gly-Gly	0
		Gly-Phe-Phe	0
		N CBZ Pro-Leu	0
		N CBZ Phe-Leu	0

* Expressed as percentage of maximal activity measured with *p*-NA substituted amino acids, dipeptides or tripeptides; * *Exprimée en pourcentage de l'activité maximale mesurée avec les acides aminés substitués (p-NA), dipeptides ou tripeptides.*

thermophilus were also found to be serine proteases (Meyer and Jordi, 1987). The enzyme was also inhibited by EDTA, thus indicating a requirement for metal ion. The same inhibition pattern was observed with the enzymes from *S thermophilus* (Meyer and Jordi, 1987). The enzyme was completely inactivated by 0.1 mM Zn and less inactivated by Co, Cu and Fe. Divalent ions Ca and Mg had no effect on the en-

Table IV. Substrate specificity of the peptidase with ß-casomorphin fragments. Hydrolysis of ß-casomorphin fragments was followed by HPLC. After incubation for 3 h at 40°C, the reactional mixture was injected onto a Vydac C 18 5 µ column and elution was performed with water/acetonitrile gradient.
Specificité de substrats de la peptidase avec les fragments de ß-casomorphine. L'hydrolyse des fragments de ß-casomorphine a été suivie par HPLC. A près une incubation de 3 h à 40 °C, le mélange réactionnel a été injecté sur une colonne Vydac C18 5µ et l'élution faite par un gradient eau/acétonitrile.

Not hydrolyzed	*Hydrolyzed*
ß-casomorphin 1-3-fragment (Tyr-Pro-Phe)	ß-casomorphin des-Tyr-fragment 7 (Pro-Phe-Pro-Gly-Pro-Ile)
Morphiceptin or ß-casomorphin 1-4 fragment (Tyr-Pro-Phe-Pro-NH2)	
ß-casomorphin 1-5 fragment (Tyr-Pro-Phe-Pro-Gly)	

zyme. The enzyme was most active at pH 8.0. This result can be compared to the broad pH optima of enzymes of *S thermophilus*: pH 6.0–8.5 (Rabier and Desmazeaud, 1973).

The optimum temperature was 40°C. The stability of the proline iminopeptidase was high, 50% of the initial activity still remaining at 50°C. The considerable activity of this peptidase at 10°C (38% of relative activity), as well as its good stability at pH 6.0 indicate the potential role that it may have during cheese ripening in the liberation of amino acids. The quantity of intracellular enzyme is quite large. However, curd conditions are unlike the experimental

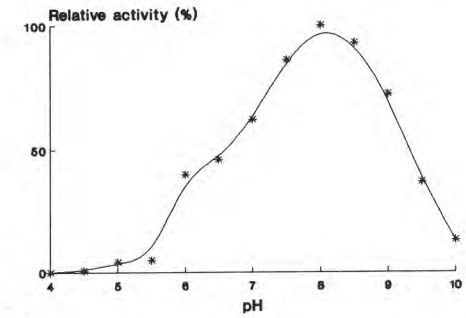

FIG.4A

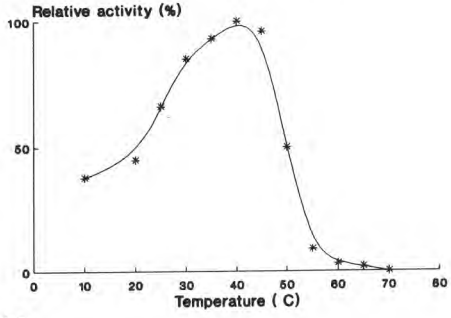

FIG.4B

Fig 4. Effect of pH (A) and temperature (B) on peptidase activity. The peptidase activity (A_{410}) was assayed with Pro *p*-NA substrate.
Role du pH (A) et de la température (B) sur l'activité de la peptidase. L'activité de la peptidase a été testée avec le substrat Pro p-*NA.*

Table V. Inhibition of peptidase activity.
Inhibition de l'activité de la peptidase.

Inhibitor	*Concentration (mM)*	*Relative activity (%)*
Dithiothreitol	0.1	100
	1	100
L-Cysteine	0.1	100
	1	90
2-Mercaptoethanol	0.1	100
	1	100
N-Ethylmaleimide	0.1	100
	1	96
Iodoacetic acid	0.1	100
	1	90
Iodoacetamide	0.1	100
	1	96
p-Chloromercuric benzoic acid	0.1	100
	1	100
EDTA	1	97
	10	62
1.10-Phenanthroline	0.1	100
	1	100
Bestatin	0.1	90
	1	75
Phosphoramidon	0.1	100
Phenylmethylsulfonyl fluoride	0.1	75
	1	30
Pepstatin A	0.1	100
	1	100
Sodium citrate	0.1	100
	1	100

conditions: uncontrolled pH values, no lysis during the exponential phase.

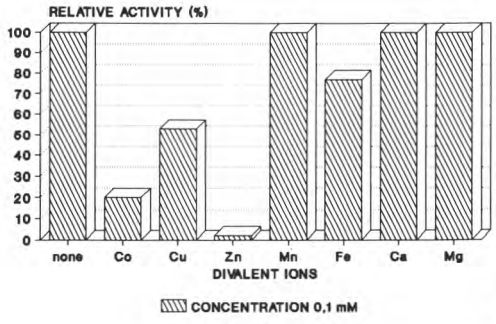

Fig 5. Effect of divalent ions on peptidase activity. The relative peptidase activity was measured by the release of *p*-nitroanilide which was followed spectrophotometrically.
Rôle des ions divalents sur l'activité de la peptidase. L'activité relative de la peptidase a été mesurée par la libération de p*-nitroanilide suivie au spectrophotomètre.*

After production of Swiss cheese, the pH is approximately 5.2 and rises to 5.6–5.8 during maturation. Also, during the ripening process the cheese is stored at different temperatures between 10–24°C. During maturation the conditions are not optimal for peptidase activity. But during all

Table VI. Amino acid composition of the proline iminopeptidase of *P shermanii* 13673. *Composition en acides aminés de la proline iminopeptidase de* P shermanii *13673.*

Amino acid	*Quantity (nmol)*	*Molar ratio (%)*
Aspartic acid/asparagine	11.1	9.3
Threonine	6.5	5.4
Serine	7.5	6.3
Glutamic acid/glutamine	7.3	6.2
Proline	2.7	2.3
Glycine	8.8	7.4
Alanine	13.7	11.5
Cysteine	2.5	2.1
Valine	9.1	7.6
Methionine	0.5	0.4
Isoleucine	4.7	3.9
Leucine	7	5.9
Tyrosine	0.3	0.3
Phenylalanine	5	4.2
Histidine	2.8	0.4
Lysine	3.6	3.6
Arginine	5.4	4.5
Tryptophan	nd	

the ripening process of 4–6 weeks, the temperature and pH values are favourable for peptidase activity. The purified enzyme catalyzed hydrolysis of dipeptide and peptide derivatives containing proline residues in the ultimate position. Pro-X peptides were specifically hydrolyzed. The X-Pro peptides were resistent to enzymatic hydrolysis. The enzyme was identified as a proline iminopeptidase also with a prolinase activity, according to the possible sequences of enzyme activities proposed by Booth *et al* (1990). Several peptidases have been isolated and characterized in *S cremoris* (Geis *et al,* 1985; Exterkate and de Veer, 1987; Van Boven *et al,* 1988), and *L lactis* (Tan and Konings, 1990). None is capable of releasing N terminal prolyl residues. Beta-casomorphin des-Tyr was a good substrate for the proline iminopeptidase of *P shermanii* 13673.

The X-prolyl dipeptidyl peptidase purified from *L lactis* (Zevaco *et al,* 1990) hydrolyzed the β-casomorphin producing 4 different peptides.

In Swiss type cheese proteinases from lactic acid bacteria provide peptides from caseins. These peptides will be further cleaved by peptidases. Peptide hydrolases from starters may include aminopeptidases with broad substrate specificities, dipeptidases, and finally leucine aminopeptidase or proline iminopeptidase in order to release essential amino acids for cell nutrition. Degradation of casein by the action of chymosin or other enzymes can result in formation and accumulation of bitter-tasting peptides in cheese (Vegarud and Langsrud, 1989).

Bitter-tasting peptides consistently have a high proportion of hydrophobic amino acid residues. Bitterness will not develop if

specific peptidolytic enzymes with debittering properties are present (Ardo *et al*, 1989). Proline-containing peptides exhibited bitterness (Ishibashi *et al*, 1988). This flavor defect may occur during accelerated ripening of cheese. Consequently, sufficient intracellular peptidases from starter cells is a critical point in the release of amino acids and debittering of dairy products. The propionibacteria also produce intracellular peptidases which are released in the cheese by autolysis (Langsrud *et al*, 1978b). Earlier observations by Langsrud *et al* (1977) suggested that proline-releasing peptidases predominate in *P shermanii*. Proline has a sweet taste and its presence in particularly high concentrations in Emmental cheese led Langsrud and Reinbold (1973) to suggest that this constituted the basis of this aspect of Swiss cheese flavor. In this paper we have described the presence of a proline iminopeptidase which enables *P shermanii* 13673 to release free proline from intermediary products of protein breakdown as observed by Langsrud *et al* (1978a).

Heat and freeze-shocking of propionibacteria can be tested to accelerate ripening of cheese, according to Frey *et al* (1986) and Bartels *et al* (1987a, b), cheese manufacturers being interested in producing mature-flavoured products in a short period of time. More research is needed to obtain strains with lytic properties as well as a high content of stable intracellular enzymes.

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