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Effect of the aminopeptidase from *Pseudomonas fluorescens* ATCC 948 on synthetic bitter peptides, bitter hydrolysate of UHT milk proteins and on the ripening of Italian Caciotta type cheese

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Summary — The activity of the purified aminopeptidase from *Pseudomonas fluorescens* ATCC 948 on synthetic bitter peptides, bitter hydrolysate of UHT milk proteins and on the ripening of Italian Caciotta type cheese was studied. The aminopeptidase almost completely hydrolyzed the bitter pentapeptide H-Leu-Trp-Met-Arg-Phe-OH and liberated Val from the bitter tetrapeptide H-Val-Pro-Leu-Leu-OH. No cleavage of the Pro-bound was observed. In UHT milk in which bitter protein hydrolysate was caused by externally added proteinase, the aminopeptidase produced a concentration of free amino acids about 8 times higher than those determined on UHT unhydrolyzed non-bitter milk (211.3 *vs* 25.3 μg/ml, respectively). Glu, Leu, Met, Trp and Val were the most abundant amino acids. The enzyme showed 44% of its maximum activity at the storage temperature of UHT milk (20°C). The aminopeptidase was stable during the ripening of Caciotta type cheese: no activity was lost during 2 months. After 45 days of ripening, the cheese containing aminopeptidase had a higher amino acid level (990.2 μg/ml) than the untreated-control (591.1 μg/ml). The amino acid profile of the treated cheese reflected the aminopeptidase activity. The specific hydrolysis of peptidic bounds involving amino acids (Leu, Trp and Val), usually identified as major components of bitter peptides, probably indicates a debittering activity of this aminopeptidase.

aminopeptidase / bitter peptide / bitter UHT milk / cheese ripening

Résumé — Effet de l'aminopeptidase de *Pseudomonas fluorescens* ATCC 948 sur les peptides amers synthétiques, les hydrolysats de protéines du lait UHT et sur l'affinage du fromage italien de type caciotta. *On a étudié l'activité de l'aminopeptidase purifiée de* Pseudomonas fluorescens *ATCC 948 sur les peptides amers synthétiques, les hydrolysats amers des protéines du lait UHT et sur l'affinage du fromage italien de type caciotta. L'aminopeptidase a presque totalement hydrolysé le pentapeptide amer H-Leu-Trp-Met-Arg-Phe-OH et libéré Val du tétrapeptide amer H-Val-Pro-Leu-Leu-OH. Aucun clivage de la liaison Pro n'a été observé. Dans le lait UHT, dans lequel*

l'hydrolyse des protéines a été provoquée par addition d'une protéinase, l'aminopeptidase a produit une concentration d'acides aminés libres environ 8 fois plus élevée que celles déterminées sur le lait UHT non hydrolysé et non amer (211,3 contre 25,3 μg/ml respectivement). Glu, Leu, Met, Trp et Val ont été les plus abondants. L'enzyme a présenté 44% de son activité maximale à la température de conservation du lait UHT (20°C). L'aminopeptidase a été stable pendant l'affinage de la caciotta: aucune activité n'a été perdue pendant 2 mois. Au terme de 45 jours d'affinage, le fromage contenant l'aminopeptidase présentait un niveau d'acides aminés plus élevé (990,2 μg/ml) que le contrôle non traité (591,1 μg/ml). Le profil des acides aminés du fromage traité reflétait l'activité de l'aminopeptidase. L'hydrolyse spécifique des liaisons peptidiques impliquant des acides aminés (Leu, Trp et Val) habituellement identifiés comme étant les principaux composants des peptides amers indique probablement une activité d'élimination de l'amertume de cette aminopeptidase.

aminopeptidase / peptide amer / lait UHT amer / affinage

INTRODUCTION

While amino acids, amines, amides, substituted amides, long chain ketones and some monoglycerides may also contribute to bitterness (Ney, 1979), it is now accepted that the bitter flavour produced in cheese during the enzymatic hydrolysis of bovine casein is due to some types of peptides (Fujimaki *et al*, 1970; Lemieux and Simard, 1991, 1992).

The occurrence of bitter flavour in UHT milk has been documented (McKellar, 1981; Sørhaug and Stepaniak, 1993) and attributed to heat-stable proteinases produced by psychrotrophic bacteria. The monitoring of raw milk refrigeration, the use of controlled or modified atmosphere storage of milk, the activation of the inhibitory system (lactoperoxidase) of milk, or the application of low temperature inactivation (LTI) (Skura, 1989) and of UHT modified treatment (Bucky *et al*, 1988) are proposed for controlling heat-stable proteinases from psychrotrophs.

Bitterness has long been recognised as a major defect in Camembert (Pélissier *et al*, 1974a), Cheddar (Edwards and Kosikowski, 1983), Gouda (Visser *et al*, 1983), and Gorgonzola (Delformo and Parpani, 1986) cheeses. A combined action of rennet and proteinases from some starter microflora is considered to be the cause of the bitter taste. Several possibilities exist for monitoring bitterness in cheese: 1) selection of starters or cheese-related microorganisms with strong peptidase and, in particular, aminopeptidase activities (Arora and Lee, 1990; El Abboudi *et al*, 1991); 2) use of mutant proteinase-negative strains and with the same peptidase activity as the parent cell (Kamaly and Marth, 1988); 3) addition of commercial mixture of proteolytic microbial and animal preparation (Fox, 1988); 4) addition of heat-treated cultures of lactic acid bacteria (Ardo *et al*, 1989); and 5) use of exopeptidases from microorganisms other than lactic acid bacteria, such as *Bacillus stearothermophilus* (Roncari and Zuber, 1970), *Brevibacterium linens* (Brezina *et al*, 1988) and *Thermus acquaticus* (Minagawa, 1989).

Following other investigations (Gobbetti and Rossi, 1992; Gobbetti *et al*, 1993, 1994), this study examines the activity of a purified aminopeptidase from *Pseudomonas fluorescens* ATCC 948 on synthetic bitter peptides, bitter hydrolysate of UHT milk proteins and on the ripening of Italian Caciotta type cheese.

MATERIALS AND METHODS

Enzyme

The aminopeptidase from *Pseudomonas fluorescens* ATCC 948 was purified by ion-exchange

chromatography (DEAE-Sephadex A-50, Pharmacia Fine Chemicals, Uppsala, Sweden), gel filtration (Biogel P-300, Biorad Labs, Richmond CA, USA), affinity chromatography (L-Leu-Gly-AH-Sepharose 4B, Pharmacia), and then lyophilized. It was characterized as metalloenzyme with broad specificity, irreversibly inactivated after a heat treatment of 75°C for 1 min and with optimal pH and temperature at 7.5 and 45°C, respectively (Gobbetti *et al*, 1994). The lyophilized preparation had a specific activity of 9.916 units/mg protein. One unit of the aminopeptidase activity was defined as the amount of enzyme needed to increase of 0.01 the absorbance at 560 nm after 1 min of incubation.

Measurement of aminopeptidase activity

The aminopeptidase activity on bitter peptides (BP), on bitter UHT milk with partially hydrolyzed proteins (BHMP) and on the ripening of Italian Caciotta type cheese was determined by the analysis of the individual free amino acids before and after the treatment with the enzyme.

Isolation of the amino acids

BHMP or Caciotta cheese extracts (5 ml) prepared as described by Desmazeaud and Vassal (1979) were centrifuged at 20 000 *g* for 20 min. One ml of the centrifuged sample (containing 0.02 to 1.2 mg/ml of DL-norleucine as double internal standard, according to the presumed amino acids content) was filtered through a Biogel P2 (400 mesh, column 1 x 60 cm, Biorad) and eluted with 0.05 mol/l phosphate buffer (pH 7.0). The fraction of eluate between 25–65 ml was collected, adjusted to pH 4 – 5 and subjected to an ion-exchange purification through an AG 50W-X8 resin (column size 1 x 12 cm) (Biorad). After washing with 45 ml of distilled water, a 40-ml fraction was eluted with 1 mol/l HCl, collected and concentrated to 5 ml under reduced pressure at 40°C. The concentrate (0.5 ml) was placed in a test tube and evaporated to dryness; the residue was derivatized.

One ml of BP was applied directly to the ion exchange column.

Derivatization of amino acids

The amino acids were transformed into isopropyl-trifluoro-acetyl derivatives according to the method of Gobbetti *et al* (1993).

Analysis of amino acids

The amino acids were separated and quantified profile was obtained by gas chromatograph (Chrompack 9001; Middelburg, EA 4330, the Netherlands) with a split-splitless injector (in split mode, split ratio was 1:30), flame ionization detector on a 25-m x 0.25-mm Chirasyl-L-Val column (Chrompack) and an EMI 80386 computer, with MOSAIC integration software (Chrompack). The column oven was temperature programmed: 60°C (15 min), to 88°C at 1.5°C/min, at 88°C (6 min), to 195°C at 2°C/min, and then held at 195°C for 15 min. Other conditions were: injector and detection temperatures, 220°C; H_2 flow rate, 20 ml/min; N_2 flow rate (make up), 20 ml/min; air flow rate, 200 ml/min; carrier gas (He) head pressure, 780 mm Hg; and injection volume, *ca* 0.1 µl.

Statistical analysis

The results are the means of six replicates (three samples, each analysed twice); the data were subjected to analysis of variance (ANOVA) (Stanton, 1988).

Aminopeptidase activity on BP

H-Leu-Trp-Met-Arg-Phe-OH and H-Val-Pro-Leu-Leu-OH (Bachem Feinchemikalien AG Products, Budendorf, Switzerland) were selected because the two peptides are part of the sequence of bitter peptides from α_{s1}-casein. Leu-Trp, corresponding to the α_{s1} f198–199 was isolated by Hill and van Leeuwen (1974), while Miniamura *et al* (1972) isolated the bitter tetrapeptide Leu-Trp-Leu-Trp. Val-Pro-Leu is the initial sequence of the bitter peptide α_{s1} f167–179 isolated by Pélissier *et al* (1974b). For the two peptides used in this study, the bitterness 'index' (Q-rule) of Ney is 2020 and

2420 cal/res, respectively. The assay mixture contained 600 µl of 0.05 mol/l potassium phosphate buffer (KPB) (pH 7.0), 200 µl of peptide solution (2.5 mmol/l final concentration) and 200 µl of aminopeptidase at a concentration of 0.05 units/ml. After incubation for 20, 40 and 60 min at 37°C, reaction was stopped with 0.1 mol/l acetic acid.

Aminopeptidase activity on BHMP

In order to obtain BHMP, UHT skim milk was treated with a neutral endoprotease B500 from *Bacillus amyloliquefaciens* (Gist Brocades, Delft, the Netherlands). This protease was shown to produce bitter peptides (Piet *et al*, 1990). On the basis of preliminary assays, the sterile enzyme solution was added aseptically (0.2 mg/ml) to UHT skim milk (pH 6.70, protein 3.2%, lactose 4.5%) for 30 min at 55°C. The endoprotease was desactivated by heating at 90°C for 10 min. An internal laboratory panel confirmed the bitter taste of the BHMP. After this treatment, aminopeptidase at a concentration of 0.01 or 0.05 units/ml BHMP was added and the mixtures were incubated at 37°C for 120 min. The reaction was stopped by TCA precipitation and the samples were centrifuged as previously described. A comparison between the activity of 0.05 units/ml BHMP at 20 and 37°C for 60 and 120 min was conducted. The activity of 0.0025 units/ml BHMP was controlled at room temperature (range 12–20°C) for 40 days (BHMP contained 0.02 w/v of Na-Azide).

The aminopeptidase activity (0.05 units/ml) was also tested at 37°C for 120 min on unhydrolyzed UHT skim milk (control).

Cheesemaking and determination of the aminopeptidase activity in cheese extract

Two batches of Caciotta type cheese were manufactured in the laboratory plant (Battistotti *et al*, 1983). Tne liters of pasteurized (16 s/72°C) whole milk supplied with 0.1 g/l of $CaCl_2$ were used for each batch. *Lactococcus lactis* subsp *lactis* F103 and *Streptococcus thermophilus* I16 belonging to the Collection of the Dairy Microbiology Institute of Perugia were added to the milk at a 2.5% ratio (v/v). After 30 min of incubation at 35°C, the coagulant consisting on calf rennet extract (Chr Hansens Laboratoty, Horsholm, Denmark) diluted 1:100 (w/v) was added to the milk at a ratio of 1:100 (v/v). After coagulation (30 min at 35°C), curd cutting (2 cm grains) and whey drainage (15°C, 18 h), the aminopeptidase (5.5 units/kg curd) was dissolved in the salt solution (8%, 3 h of salting) and added to the milled curd of the first batch of cheese while the second batch was untreated and considered as the control. The heating of Caciotta type cheese at 50°C was excluded in order to avoid loss of the enzymatic activity. The ripening was conducted for 2 months at 10°C (80% of relative humidity).

The aminopeptidase stability in the cheese was measured in the water soluble extract of cheeses prepared by the method of Desmazeaud and Vassal (1979). Aminopeptidase activity of the supernatants was measured by using Leu-βna-naphthylamide as a substrate (Gobbetti *et al*, 1994).

Cheese analysis

The pH was recorded in diluted (1:10) and homogenized samples. The total solids of cheese were determined on 5 g of grated cheese at 103°C ± 2°C for 15 h. Salt was determined by potentiometric method (Fox, 1963). Total protein and non-protein nitrogen (NPN) were determined by the micro-Kjeldahl method. The NPN was obtained by adding trichloroacetic acid (12% final concentration) to the diluted (1:10) and homogenized samples.

RESULTS AND DISCUSSION

Effect on bitter peptides (BP)

The aminopeptidase from *Pseudomonas fluorescens* ATCC 948 completely hydrolyzed the H-Leu-Trp-Met-Arg-Phe-OH pentapeptide. Complete hydrolysis of Leu (2.5 mmol/l represented 100% hydrolysis) was recorded after 20 min of incubation (fig 1), almost all Trp (2.45 mmol/l) and about 60% of the Met were released after 40 min. High activity on Leu at N-terminal confirmed the

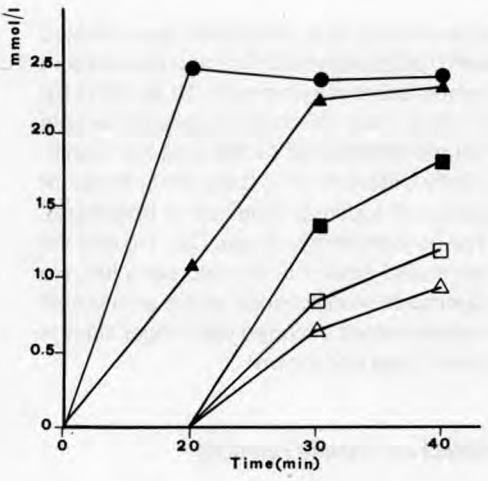

Fig 1. Leu (●), Trp (▲), Met (■), Arg (□) and Phe (Δ) concentrations after hydrolysis of the pentapeptide H-Leu-Trp-Met-Arg-Phe-OH by the aminopeptidase from *Pseudomonas fluorescens* ATCC 948.
Concentrations de Leu (●), *Trp* (▲), *Met* (■), *Arg* (□) *et Phe* (Δ) *après hydrolyse du pentapeptide H-Leu-Trp-Met-Arg-Phe-OH par l'aminopeptidase de* Pseudomonas fluorescens *ATCC 948.*

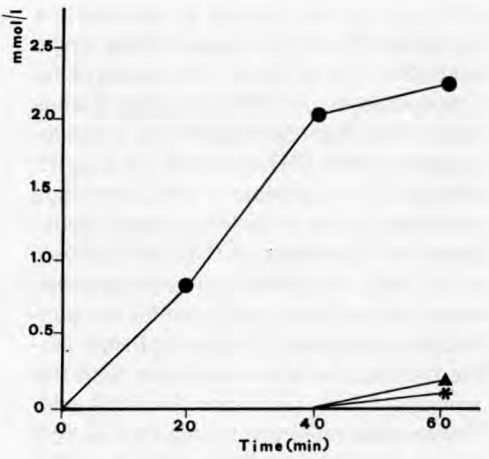

Fig 2. Val (●), Pro (▲) and Leu (✳) concentrations after hydrolysis of the tetrapeptide H-Val-Pro-Leu-Leu-OH by the aminopeptidase from *Pseudomonas fluorescens* ATCC 948.
Concentrations de Val (●), *Pro* (▲) *et Leu* (✳) *après hydrolyse du tétrapeptide H-Val-Pro-Leu-Leu-OH par l'aminopeptidase de* Pseudomonas fluorescens *ATCC 948.*

previously determined specificity of this enzyme (Gobbetti *et al*, 1994).

The hydrolysis of Val from the BP H-Val-Pro-Leu-OH was almost complete after 20 min (fig 2). However, only traces of Pro and Leu were detected after 3 h of treatment with aminopeptidase. The incapacity of this enzyme to cleave bound peptides involving Pro residue was confirmed (Gobbetti *et al*, 1994).

Effect on bitter hydrolyzed of UHT milk proteins (BHMP)

As used by others (Minagawa *et al*, 1989; Piet *et al*, 1990), the first step for studying the activity of the aminopeptidase on BHMP was to produce bitter peptides using *B amyloliquefaciens* neutral endoprotease B500. BHMP contained a significantly ($P < 0.05$) higher concentration of free amino acids than the untreated UHT skim milk control (53 μg/ml, sample 2, *vs* 10.4 μg/ml, sample 1). The extensive hydrolysis was due to endoprotease B500 and probably to a contamination of the commercial enzyme with aminopeptidase. After heating (90°C for 10 min) BHMP, the concentration of free amino acids did not increase during 120 min at 37°C (60 μg/ml, sample 4). The heating was necessary to avoid residual endoprotease B500 activity at 37°C (the free amino acid concentration increased from 53 μg/ml, sample 2, to 82 μg/ml, sample 3). The incubation of UHT skim milk at 55°C for 30 min and then at 37°C for 120 min did not significantly modify the amino acid concentration of sample 1 (data not shown). Treatment of BHMP with pseudomonad aminopeptidase (0.05 units/ml BHMP) at

37°C for 120 min (sample 8) resulted in a significant ($P < 0.05$) increase of free amino acids (211.3 *vs* 53 μg/ml). The activity of the aminopeptidase on BHMP was about 8 times higher than that observed on the unhydrolyzed non-bitter UHT skim milk (25.3 μg/ml, sample 5). In agreement with a previous characterization of cell-associated peptidases of *P fluorescens* ATCC 948 (Gobbetti *et al*, 1993), the BHMP can also be considered as a suitable substrate for the purified aminopeptidase. Comparing BHMP profile before and after treatment with the aminopeptidase, significant ($P < 0.05$) differences were observed for Ala (3.8 *vs* 12.5 μg/ml), Glu (10.2 *vs* 55.6 μg/ml), Leu (3.8 *vs* 51.2 μg/ml), Met (0.1 *vs* 11.5 μg/ml), Trp (3.5 *vs* 23.6 μg/ml) and Val (3.5 *vs* 18.4 μg/ml). The increased concentration of Leu, Trp and Val reflected the specificity shown by synthetic BP. The specific accumulation of Leu, Met, Trp and Val, which usually appear as the major components of bitter peptides, probably indicated debittering of BHMP. As postulated by the Q rule (Ney, 1979), the liberation of only one strongly bitter amino acid (Leu and Trp have high Q values of 2420 and 3000 cal/res, respectively) from a bitter sequence could generate a new non-bitter peptide. At 20°C (sample 9) the activity of the aminopeptidase at concentration of 0.05 units/ml BHMP also produced a significant ($P < 0.05$) increase of the amino acid content of BHMP (from 53 to 90.4 μg/ml) (table I). The activity at this temperature was 44% of the maximum activity of this enzyme. Gobbetti *et al* (1994) determined that the enzyme shows 50% and 100% activity on Leu-βna at 20 and 37°C, respectively. An increase either of the amount of enzyme used from 0.01 to 0.05 units/ml BHMP (samples 6 and 7) or a longer incubation time from 60 to 120 min (samples 7 and 8) significantly increased ($P < 0.05$) the final concentration of the free amino acids.

To determine the potential activity of the aminopeptidase during the long-term storage of UHT milk, the BHMP were treated with 0.0025 units/ml BHMP and kept at room temperature varying from 12 to 20°C for 40 days. After 25 days of storage (sample 10) the amino acid profile was not significantly different ($P < 0.05$) from those of sample 8 (optimal condition of treatment). The concentrations of Leu, Glu, Trp and Val were also similar in the two samples. No significant modifications of the amino acid concentrations occurred with longer storage times (data not shown).

Effect on cheese ripening

The moisture, protein, NPN (expressed as percentage of NPN/total nitrogen), salt and pH of the two cheeses produced are shown in table II. The levels were within normal limits for Caciotta cheese (Battistotti *et al*, 1983). There was no significant ($P < 0.05$) difference in cheese composition between the experimental and control batches after 1 day. The level of NPN increased from about 6% in the 1-day-old cheeses to about 14.5% after 60 days of ripening (fig 3). The content and rate of the NPN increase during ripening were not significantly ($P < 0.05$) dependent on the addition of aminopeptidase during manufacturing. However, the results illustrated in figure 3 indicate that the aminopeptidase-treated Caciotta type cheese exhibited a higher aminopeptidase activity throughout the ripening period. The level of aminopeptidase activity in the extract-treated cheese remained constant during 2 months. In contrast, the control cheese showed a low aminopeptidase activity on Leu-βna after only 15 days of ripening. The time necessary to reach this aminopeptidase activity could correspond to the extensive lysis of lactic acid bacteria starters during the first or second weeks of the ripening period (Law and Sharpe, 1975). Even though only 35% of the maximum activity was retained in acidic buffer (Gobbetti *et al*,

1994), the stability of the aminopeptidase during Caciotta type ripening could have increased due to the divalent cations present in the cheese (Jarret, 1979). Confirming the important role of the intracellular aminopeptidases of lactic acid bacteria during cheese ripening (El Abboudi *et al*, 1991), the amino acid profile of experimental and control Caciotta type cheese significantly ($P < 0.05$) differed after 45 days of ripening. The aminopeptidase from *P fluorescens* ATCC 948 caused a higher accumulation of free amino acids (990.2 *vs* 571.1 µg/ml). Compared to the not detec-

Table I. Free amino acid concentration in the 10 analyzed samples.
Concentration d'acides aminés libres dans les 10 échantillons analysés.

Amino acids	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
					µg/ml					
Ala	0.5	3.8	5.9	4.2	1.1	5.4	7.6	12.5	5.3	16.3
Asp	0.1	0.2	1.3	0.1	1.7	2.0	2.1	5.5	1.8	7.2
Cys	0.1	0.1	0.5	0.1	0.1	0.8	1.2	0.7	1.3	0.5
Gly	1.9	3.1	3.6	3.8	2.1	1.8	1.5	0.8	0.4	0.8
Glu	1.3	10.2	18.6	12.2	2.7	22.5	31.1	55.6	17.4	60.4
Ile	0.2	0.6	0.8	0.8	0.3	1.8	1.4	1.7	1.6	1.0
Leu	0.6	3.8	3.5	3.8	3.6	18.8	25.5	51.2	23.2	58.7
Lys	0.2	7.5	12.3	8.9	1.0	0.7	1.3	1.9	6.8	2.2
Met	1.0	0.1	0.3	0.1	1.8	3.5	4.9	11.5	2.7	10.8
Phe	0.5	6.8	10.3	7.9	1.8	0.5	2.5	4.7	1.0	3.8
Ser	0.7	5.3	7.2	5.3	1.3	0.2	1.0	9.1	2.1	7.4
Thr	0.5	2.2	3.5	3.4	1.3	1.5	2.8	4.3	1.7	3.7
Tyr	1.4	4.0	7.4	4.1	2.5	1.5	2.8	9.8	2.1	4.8
Trp	0.5	1.8	1.6	2.1	2.8	14.8	20.3	23.6	15.8	22.2
Val	0.9	3.5	5.2	3.2	2.2	6.5	10.8	18.4	7.2	21.3
Total	10.4 [a]	53.0 [b]	82.0 [c]	60.0 [b]	25.3 [d]	82.3 [c]	116.8 [e]	211.3 [f]	90.4 [c]	221.1 [f]

[a,b,c,d,e,f] Items in the total amino acids row followed by different superscript letters differ ($P < 0.05$). 1, UHT skim milk; 2, UHT skim milk treated with B500 for 30 min at 55°C (BHMP); 3, UHT skim milk treated with B500 for 30 min at 55°C and then incubated for 120 min at 37°C; 4, UHT skim milk treated with B500 for 30 min at 55°C and then incubated for 120 min at 37°C after heat-treatment; 5, UHT skim milk treated with aminopeptidase (0.05 units/ml) from *Pseudomonas fluorescens* ATCC 948 for 120 min at 37°C; 6, BHMP treated with aminopeptidase (0.01 units/ml) for 60 min at 37°C; 7, BHMP treated with aminopeptidase (0.05 units/ml) for 60 min at 37°C; 8, BHMP treated with aminopeptidase (0.05 units/ml) for 120 min at 37°C; 9, BHMP treated with aminopeptidase (0.05 units/ml) for 120 min at 20°C; 10, BHMP treated with aminopeptidase (0.0025 units/ml) for 25 days at room temperature.

[a,b,c,d,e,f] *Les items de la série totale des acides aminés suivis de lettres distinctes diffèrent (P < 0,05). 1 = lait UHT écrémé ; 2 = lait UHT écrémé soumis à l'action de B500 pendant 30 min à 55°C (BHMP) ; 3 = lait UHT écrémé soumis à l'action de B500 pendant 30 min à 55°C, puis incubé pendant 120 min à 37°C ; 4 = lait UHT écrémé soumis à l'action de B500 pendant 30 min à 55°C, puis incubé pendant 120 min à 37°C après le traitement thermique ; 5 = lait UHT écrémé soumis à l'action de l'aminopeptidase (0,05 unités/ml) de* Pseudomonas fluorescens ATCC 948 *pendant 120 min à 37°C ; 6 = BHMP soumis à l'action de l'aminopeptidase (0,01 unités/ml) pendant 60 min à 37°C ; 7 = BHMP soumis à l'action de l'aminopeptidase (0,05 unités/ml) pendant 60 min à 37°C ; 8 = BHMP soumis à l'action de l'aminopeptidase (0,05 unités/ml) de pendant 120 min à 37°C ; 9 = BHMP soumis à l'action de l'aminopeptidase (0,05 unités/ml) pendant 120 min à 20°C ; 10 = BHMP soumis à l'action de l'aminopeptidase (0,0025 unités/ml) de pendant 25 jours à température ambiante.*

Table II. Composition (%) of Caciotta type cheeses made traditionally (control) and with the addition of the aminopeptidase from *Pseudomonas fluorescens* ATCC 948.
Composition (%) de fromages du type Caciotta fabriqués de façon traditionnelle (contrôle) et avec addition de l'aminopeptidase de Pseudomonas fluorescens *ATCC 948.*

Cheese type	*Moisture*	*Protein*	*NPN/TN*	*Salt*	*pH*
Control	36.35	26.38	5.90	1.82	5.31
Aminopeptidase addition	36.82	26.75	6.10	1.72	5.28

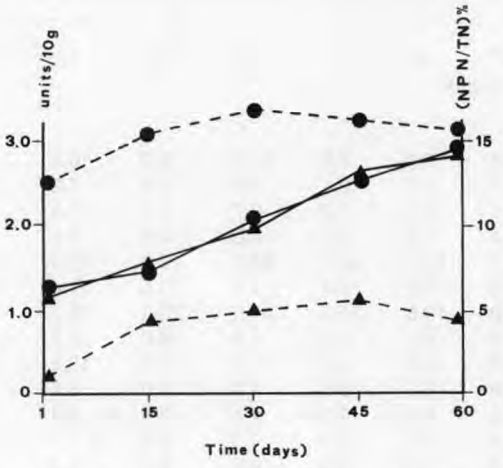

Fig 3. Stability of the aminopeptidase activity (units/10 g of d.w. cheese) (– – –) and evolution of NPN/TN (%) (——) in Caciotta type cheese made traditionally (control) (▲) and with the addition of aminopeptidase from *Pseudomonas fluorescens* ATCC 948 (●).
Stabilité de l'activité de l'aminopeptidase (unités/10 g de fromage pds sec) (– – –) *et évolution du rapport azote non protéique / azote total (%)* (——) *dans le fromage du type Caciotta fabriqué de façon traditionnelle (contrôle)* (▲) *et avec addition de l'aminopeptidase* (●) *de* Pseudomonas fluorescens *ATCC 948.*

table influence of the aminopeptidase on the NPN, the increase of free amino acids content can be attributed either to differences on the sensitivity of the two analytical methods or to the relevant hydrolysis of peptides (included on NPN) to produce free amino acids. No significant variations were observed after further 15 days of ripening (data not shown). As far as the aminopeptidase activity on BHMP is concerned, the highest differences were attributed to Glu (213.7 *vs* 126.5 μg/ml), Leu (306.2 *vs* 198.9 μg/ml), Met (65.7 *vs* 33.6 μg/ml), Trp (79.7 *vs* 37.8 μg/ml) and Val (77.6 *vs* 42.0 μg/ml). This specific aminopeptidase activity during cheese ripening indicated the potential to hydrolyze peptides and probably bitter peptides generated by the combined action of rennet and bacterial proteolytic enzymes. Use of non-starter aminopeptidase preparations to reduce bitterness in cheese was proposed earlier (Roncari and Zuber, 1970; Brezina *et al*, 1988; Minagawa, 1989). Malkki (1978) and Malkki *et al* (1979) found that a strain of *P fluorescens* produced a complex of endopeptidases and exopeptidases that converted 60 to 90% of the milk proteins to free amino acids. In particular, bitterness was absent when peptidases from *P fluorescens* VTTE 1.8 were added to Edam-type cheese (Malkki, 1978); the enzyme preparation also prevented bitterness and enhanced the ripening process of Cheddar-type cheese made with calf rennet (Malkki *et al*, 1979). Our studies indicated that aminopeptidase from *P fluorescens* ATCC 948 has promising specificity for degrading bitter peptides.

Presence of D-amino acid isomers was reported for various dairy products and was also attributed to microbial activity (Bruckner and Hausch, 1990; Gobbetti *et al*, 1994). In

Table III. Amino acid profiles of Caciotta type cheeses made traditionally (control) and with the addition of aminopeptidase from *Pseudomonas fluorescens* ATCC 948 during ripening.
Profils des acides aminés de fromages du type Caciotta fabriqués de façon traditionnelle (contrôle) et avec addition de l'aminopeptidase de Pseudomonas fluorescens *ATCC 948 pendant l'affinage.*

Amino acids	*1*	*2*	*3*	*4*
			µg/ml	
Ala L	2.1	2.8	7.6	22.7
D			8.5	8.0
Asp L	1.2	1.0	9.4	7.1
D			1.6	0.9
Cys	0.1	0.1	0.3	0.3
Gly	3.2	2.5	11.6	18.7
Glu L	7.8	8.7	106.5	213.7
D			14.5	11.3
Ile	1.2	1.6	6.0	18.9
Leu	8.7	10.2	178.9	306.2
Lys L	0.2	0.7	44.4	38.8
D			39.4	41.2
Met L	0.7	0.7	33.6	65.7
D				0.5
Phe L	8.1	6.9	3.8	11.9
D			5.2	3.7
Ser	0.7	2.1	12.8	23.8
Thr	1.2	1.3	7.6	12.3
Tyr L	0.4	0.6	10.2	14.3
D			9.4	12.9
Trp	1.8	1.9	27.8	79.7
Val	2.6	3.4	32.0	77.6
Total	40.0 [a]	44.5 [a]	571.1 [b]	990.2 [c]

[a,b,c] Items in the total amino acids row followed by the different superscript letters differ ($P< 0.05$). 1, control at 1 day; 2, cheese with aminopeptidase at 1 day; 3, control at 45 days; 4, cheese with aminopeptidase at 45 days.
[a,b,c] Les items de la série totale des acides aminés suivis de lettres distinctes diffèrent (P< 0,05). 1 = contrôle à 1 jour ; 2 = fromage avec aminopeptidase à 1 jour ; 3 = contrôle à 45 jours ; 4 = fromage avec aminopeptidase à 45 jours.

particular, average concentrations of D-Ala (8.2 µg/ml), D-Glu (12.9 µg/ml), D-Lys (40.3 µg/ml) and D-Tyr (11.1 µg/ml) were detected in control and experimental Caciotta type cheese, indicating that the addition of pseudomonad aminopeptidase did not affect the isomerization process.

The broad specificity, the high activity on bitter peptides with hydrophobic amino acids at the N terminus, and the stability to acidic conditions propose the aminopeptidase from *P fluorescens* ATCC 948 as a debittering enzyme to use as additive in various dairy produtcs. Because of the ability of *Pseudomonas* spp proteinases to generate bitter peptides and because of the physiological ability of the peptidases of the same microorganism to cleave the peptides produced by its own proteinases, the aminopeptidase considered in this study may have a specific aptitude to remove the bitter flavour in milk products. Genetic engineering or

technological solutions must be considered in order to use this heat-sensitive aminopeptidase in UHT milk.

Further work is currently being carried out in order to evaluate the treated dairy products by sensory analysis and to determine a chemometric model for the aminopeptidase application.

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