DEBITTERING OF TRYPTIC DIGESTS FROM β-CASEIN AND ENZYME MODIFIED CHEESE BY X-PROLYL DIPEPTIDYLPEPTIDASE FROM *LACTOBACILLUS CASEI* SSP. *CASEI*. LLG[*]

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Abstract – The proline-rich β-casein was digested *in vitro* with trypsin, and the oligopeptides produced were then isolated by RP-HPLC and subsequently identified by amino acid analysis and ion mass spectrometry. The peptide fractions from the complete digestion were then treated with purified x-prolyl dipeptidyl peptidase (X-PDP) extracted from *Lactobacillus casei* ssp. *casei* LLG. Two bitter peptides (f53-97 and f203-209) containing X-Pro-Y-Pro in their amino acid residues were completely hydrolyzed by X-PDP, while several peptides with a high degree of hydrophobicity were also decreased in a peak area. The debittering effect of X-PDP from *Lactobacillus casei* ssp. *casei* LLG on enzyme modified cheese (EMC) was also investigated by both subjective and objective methods. The bitterness of cheddar cheese slurries supplemented with Neutrase® 0.5 L was completely eliminated after treatment with crude enzyme extract from *Lactobacillus casei* ssp. *casei* LLG. Two hydrophobic peptides in EMC with Ala-Pro-Phe-Pro-Glu-Val and Phe-Leu-Leu residues were hydrolyzed by crude enzyme extract. The RP-HPLC, and subsequently, ion mass spectrometry analysis have shown that the debittering effect on EMC was due partially to the presence of X-PDP.

Keywords – Debittering, β-casein, enzyme modified cheese, X-Prolyl Dipeptidyl Peptidase, lactobacilli

1. INTRODUCTION

Although *Lactobacillus casei* and their subspecies are not used as starter cultures during the manufacture of most types of cheese, the bacteria are commonly isolated from a fairly wide variety of ripened cheese [1]. They are believed to play an important role in the ripening of related hard cheese. The complex proteolytic enzyme systems of starter and non-starter lactic acid bacteria can complementarily act with rennet and/or indigenous milk proteinases in different phases of the long term process of cheesemaking and ripening. The overall reactions lead to a breakdown of casein into small peptides, amino acids, and primary as well as secondary products of metabolism. These components are used as a nitrogen source for the starter growth as well as render cheese the characteristic flavor/aroma [2] and indeed under certain conditions, flavor defect such as bitterness [3, 4].

The mechanisms by which bitter peptides from milk casein are removed by wheat carboxypeptidase [5], aminopeptidaseT [6], aminopeptidase II [7], aminopeptidase N [8], and with endopeptidase [9] have been comprehensively reported. Basically, exopeptidases that are able to hydrolyze proline-containing peptides play a very important role, either by direct degradation of proline-containing peptides that often

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possess a bitter taste [10] or indirectly by removing the blockage (proline residue) that blocked further degradation of such peptides by general aminopeptidases [11], therefore, it is important to identify the peptidases that are responsible for debittering the bitter peptides in cheese and thus avoiding the development of bitterness.

Enzyme modified cheese (EMC) supplemented with Neutrase[®]0.5L (neutral protease derived from *Bacillus subtilis*), tends to induce accumulation of intense bitter peptides [12, 13]. This accumulation could be reduced by further treatment of the slurries with peptidases. When Neutrase[®]0.5L and the crude enzyme extract from *Lactobacillus casei* ssp. *casei* LLG were added to the young or unripened cheese slurry followed by a controlled incubation process, an intense cheese flavor without bitterness was developed in three days [14]. In further investigation, X-prolyl dipeptidyl peptidase activity has been detected in the crude extract of *Lactobacillus casei* ssp. *casei* LLG and was purified to homogeneity and characterized [15]. The enzyme has the ability of liberating amino acid residue containing proline at penultimate position to N-terminal. This study was carried out to investigate the role of X-PDP from *Lactobacillus casei* ssp. *casei* LLG on debittering the tryptic digest of β-casein and to explain the way in which debittering activity on bitter EMC might occur (using previous data obtained in our laboratory) [14, 16].

2. MATERIALS AND METHODS

a) Chemicals, Enzymes, and Equipment

Unless otherwise specified, all substrates and other chemicals used in this study were purchased from Sigma Co. (St. Louis, USA). Triflouroacetic acid (TFA) was purchased from Aldrich Chemical Co. (Wis. USA) and acetonitrile (HPLC grade) from Caledon Laboratories Ltd. (Ontario, Canada). The HPLC system used for separation of peptides consisted of a 600E system controller, a U6K injector, a 486 tunable absorbance detector, a fraction collector, and a millenium 2010 chromatography manager. The column used was Delta pack C-18 (30 x 150mm). X- prolyl dipeptidyl peptidase was isolated and purified from *Lactobacillus casei* ssp. *casei* LLG as described previously [15].

b) Hydrolysis of β-casein

Portions (1 mg/ml) of pure β-casein were suspended in 0.05 *M* sodium phosphate buffer (pH 7.0) and incubated at 45°C with 20 μg of trypsin (234 U/ mg casein) for 18 h. The digests were inactivated by placing them in hot water (80°C) for 10 min. The samples were then cooled, passed through a 0.45μm syringe filter (Nippon, Millipore, Japan) and were further hydrolyzed with 12.5 μl (25.5 U/mg protein) of the purified X-PDP. The mixtures were incubated at 30 °C for 6, 12, and 24 h and subsequently heated at 80°C for 10 min to stop the reaction. The mixture was then cooled, lyophilized in a Speed-Vac (Savant Instruments, NY, USA) and stored at -20°C for further analysis.

c) Hydrolysis of Enzyme-Modified Cheese

EMC was prepared according to the method of Park *et al* [14]. Shredded fresh cheddar cheese (600 g) was mixed with 2.5% phosphate salt (Na_2HPO_4) and 195 ml of distilled water.The mixture was heated at 90°C for 3 min and cooled down to 50°C. Neutrase[®](.05 ml; proteinase activity 1983.33 unit/ml), was passed through 0.22 μm membrane filter and was added to the cheese slurry. The slurry was incubated for 24 and 96 h (designated N24 and N96, respectively) under vacuum at 45°C. The slurry hydrolysates of N24 and N96 were then transferred to sterilized Erlenmeyer flasks where the crude enzyme extract of *Lactobacillus casei* ssp. *casei* LLG were added and the mixture was incubated for 72 h at 34°C. To prepare the water-soluble peptides (WSP) of EMC, portions (5 g) of EMC were centrifuged (15000 x *g*, 50 min) at

20°C. A portion of supernatant (1 ml) was mixed with 1ml methanol,1ml methylen chloride, and 0.6 ml water and mixed well by vortex. After centrifugation (15000 x *g* for 30 min), 1 ml of methanol-water fraction was dried by a Speed-Vac for 3 h and reconstituted with 50 μl of distilled water.

d) High Performance Liquid Chromatography Analysis

Fractionation and separation of peptides derived from proteolytic digestion of β-casein and their hydrolysates due to the action of X-PDP, as well as those produced in WSP after treating with the crude extract, was accomplished using analytical RP-HPLC. Portions (25μl) of the tryptic digest (10% w/v) of the above samples were injected on a Delta pack C_{18} column. The peptides were eluted with a gradient of binary solvents from 0 to 100% at a flow rate of 0.5 ml/min for 60 min by using a system controller. Solvent A was Milli Q water with 0.1% TFA and solvent B was 0.8% TFA in acetonitrile-water (40:60). Eluted peptides at A_{214} were collected and lyophilized.

e) Identification of Peptide Fractions

Identification of the peptides was carried out by analyzing the amino acid composition according to the general method of Spackman *et al* [17], modified by Veeraragavan *et al* [18]. The lyophilized peptide fractions were transferred into Corning culture tubes (6 x 50 mm) which were previously heated at 450°C overnight. After placing the tubes in Waters reaction vials and drying them in the Waters Pico-Tag work station (Waters, CT, USA), constant boiling HCl (200μl) containing 1% phenol was added to the vials and alternately purged with dried nitrogen and evacuated. The hydrolysates were dissolved in 0.1 *M* sodium citrate buffer (pH 3) prior to applying to the Beckman system 6300 High Performance Analyzer. Identification was also carried out by liquid chromatography/mass spectrometry (LC/MS) at the Biotechnology Research Institute (BRI, Montreal). Mass spectra were obtained in the positive mode on a triple stage mass spectrometer model API III (Scix, Toronto, Canada). The samples were dissolved in 10% acetic acid and infused through a stainless steel capillary column (10 μm ID). A stream of air (pneumatic nebulization) was introduced to assist the formation of submicron droplets. These droplets were evaporated at the interface by nitrogen gas producing highly charged ions, which were then detected by the analyzer. The system's calibration was performed with the ammonium adduct ions of polypropylene glycol (PPG) with known mass to charge ratios throughout the range of the instrument (0-2470 amu). Simple algorithms correlate the ratio of charges produced by the compounds to their molecular weights [19].

f) Sensory evaluation

The taste panel consisted of five persons who organoleptically evaluated the bitterness of EMC. Judges were selected by their ability to differentiate the degree of bitterness using sequential triangle tests on dairy products [20]. EMC prepared with Neutrase®0.5L for 24 h (N24) was chosen as standard enzyme modified cheese and two EMC samples designated as N96 (prepared with Neutrase®0.5L for 96 h) and NL72 (further treatment of N96 with crude extract for 72 h) were compared to the standard. Quinine sulfate was used as a bitter standard according to Minagawa *et al* [6], by which a range of solutions with increasing concentrations were prepared.

3. RESULTS AND DISCUSSION

a) Hydrolysis of β-casein by trypsin

The RP-HPLC profile of tryptic digest of β-casein is shown in Fig. 1A. The identification of major peptide-containing fractions was accomplished by analyzing proteolytic fragments of β-casein released by

trypsin using mass-spectrometry. Identification of the peptides by mass-spectrometry was found to be more efficient than the amino acid composition analysis [8]. The results obtained by RP-HPLC fractionation agreed with the fragmentation pattern obtained by Tan *et al* [8], Carles and Ribadeau-Dumas [21], and Leadbeater and Ward [10]. However, the difference noticed here might be due to different gradient elution conditions employed. The results of the identification of the β-casein fragments are summarized in Table 1. The numbers of the identified fragments are as shown in Fig. 1A.

Elution time (min)
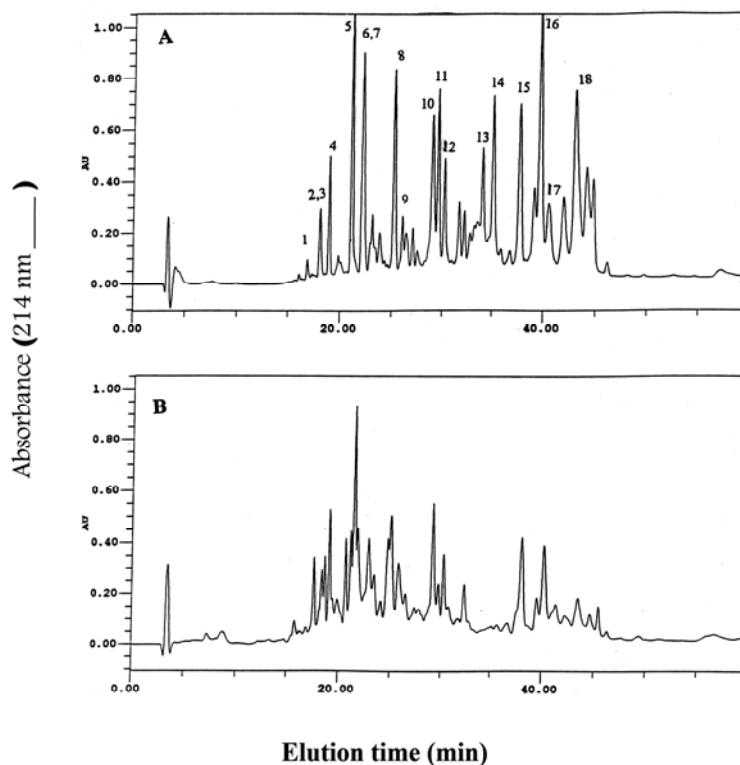
Fig. 1. Elution profile of tryptic digest of β-casein (A) and their hydrolysate with X-prolyl dipeptidyl peptidase from *Lactobacillus casei* ssp. *casei* LLG (B) using RP-HPLC

Table 1. The effect of X-PDP on peptides identified in a tryptic digest of β-casein

Peptides Identification			Effect of X-PDP on peak
Peaks	**Fragments**	**Q-value**[*]	
1	26-29	1490	Decreased
2	30-32	1673	Decreased
3	164-169	657	Decreased
4	100-105	1238	Increased
5	177-183	1594[**]	Increased
6	33-48	583	Increased
7	170-176	1870[**]	Increased
8	108-113	1873**	Not changed

Table 1. (Continued)

9	49-52	1897	Increased
10	191-202	1621	Decreased
11	1-25	1004	Decreased
12	53-68	1505**	Decreased
13	164-183	1484**	Decreased
14	203-209	2217**	Disappeared
15	71-97	1512**	Decreased
16	184-202	1581	Decreased
17	53-97	1511**	Disappeared
18	114-169	1382	Disappeared

*Q value (cal/mole) calculated according to Ney [22].
**Fragments with X-Pro-Y-Pro residues

b) Hydrolysis of tryptic β-casein hydrolysate by purified X-PDP

The elution of the tryptic β-casein digest after 12 h incubation with purified X-PDP (Fig. 1B) revealed that several peaks from the hydrophobic region (Fig. 1A) had disappeared or decreased. As shown in Table 1, all of these peptides seem to have X-Pro-Y-Pro in their amino acid residues. The results clearly show that peak 14 (f203-209), which had the highest Q-value and peaks 17 (f53-97) and 13 (f164-183) with a similar Q-value of 17, reduced in area and contained X-Pro-Y-Pro. In general, when the Q-value of a peptide is higher than 1400 cal/mol a peptide is bitter [22].

c) Treatment of Enzyme Modified Cheese (EMC)

Figures 2A, and 2B showed the elution profiles of EMC slurries supplemented with Neutrase®0.5L after 24 and 96 h incubation, respectively. It can be observed that the longer the incubation time, the more fragments produced. When N96 (96 h incubated EMC) was treated with crude enzyme extract of *Lactobacillus casei* ssp. *casei* LLG, two peaks (peaks I and II in Fig. 2B) eluted at 37.96 and 41.36 min completely disappeared (Fig. 2C). The sensory evaluation of different trials of EMC (N24, N96, and NL72) is shown in Table 2. These results clearly indicate the debittering effect of *Lactobacillus casei* ssp. *casei* LLG on EMC as no bitterness was detected in the samples. Amino acid analyzer identified these two peaks. Peak I with 6 amino acids (Ala-Pro-Phe-Pro-Glu-Val) was identified as one fraction (f26-31) of α_{s1}-casein, and peak II with 3 amino acids (Phe-Leu-Leu) was identified as one fraction (f190-192) of β-casein (Table 3). Peak I (f26-33) was found to be one of the bitter peptides derived from the hydrolysis of casein [3, 23, and 24]. As the ability of purified X-PDP enzyme to hydrolyze X-Pro-Y-Pro was demonstrated, it is reasonable to relate the removal of peak I to the action of this enzyme. The peptide of peak I (α-CN f26-31), generated by the broad specificity of Neutrase®0.5L, showed a high Q-value (1810), which is more evidence to prove that this peptide had a significant contribution to the bitterness of EMC N96. The peptide of peak II (β-CN f190-192) has also been reported to be one of the bitter peptides released by the proteolysis of bovine casein with Q-value 2496.7 [25]. It is possible that these two hydrophobic peptides from EMC (peak I and II) hydrolyzed by the concerted action of general

aminopeptidase and proline –specific peptidases including X-PDP as demonstrated by Smid *et al* [26]. Studies on purified aminopeptidase from *Lactobacillus lactis* ssp. *Cremoris* Wg2 [8] and *Lactobacillus casei* ssp. *casei* LLG [16] confirmed that aminopeptidase was unable to hydrolyze the structure of X-Pro-Y-Pro. The crude enzyme extract of *Lactobacillus casei* ssp. *casei* has been used successfully in the acceleration of cheddar cheese aging without the development of bitterness [27, 28]. Understanding the mechanism of these processes may lead to the development of a debittering enzyme in cheese and other protein hydrolysates. Apart from Q-value, the amino acid sequence of peptides is an important factor determining the degree of bitterness [29, 30]. Further studies are needed to identify the amino acid sequence of the peptides as well as the quantitative relationship between the structure and concentration of individual peptides and the intensity of bitterness of mixture peptides.
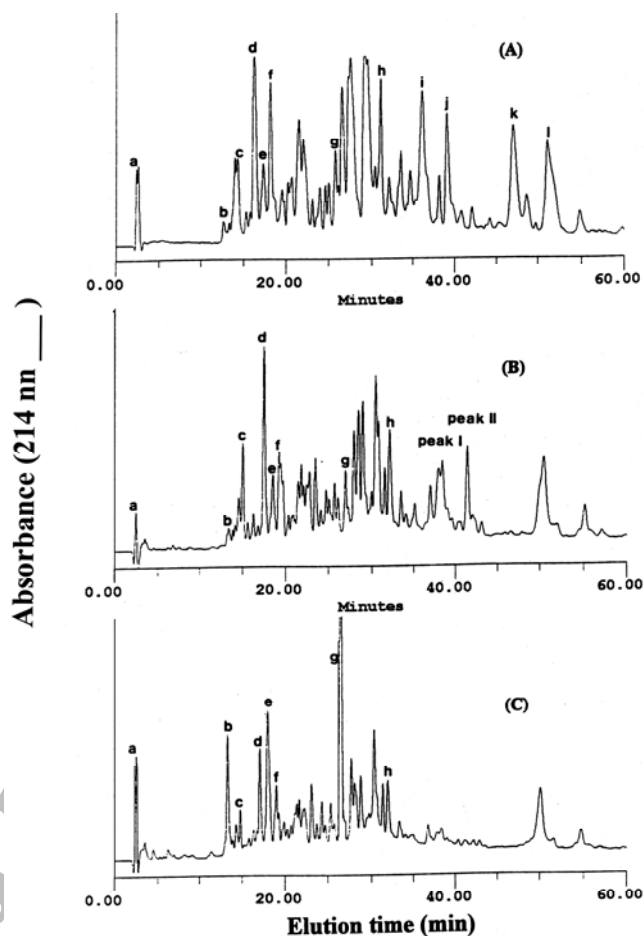

Fig. 2. RP-HPLC chromatogram of EMC N24 (A), EMC N95 treated with Neutrase 0.5L (B), and EMC N96 treated further with crude enzyme extract of *Lactobacillus casei* ssp. *casei* LLG (C)

Table 2. Sensory evaluation of EMC prepared with (NL 72) and without (N 96)
treatment by crude enzyme extract of *Lb. casei* ssp. *casei* LLG

Type of EMC	Bitter score[1]
EMC N24	4.3 (0.64)
EMC N96	6.4 (0.95)
EMC NL72	0.2 (0.32)

[1]The intensity of the bitter taste expressed on a scale of 0-8 (0=not observed,
2=slightly bitter, 4=distinctly bitter, 6=strong bitter, 8=very strong bitter)
The scores are the mean of three samples with their standard deviation in parenthesis

Table 3. Amino acid composition of peak I and II isolated from bitter EMC (N96)

Amino acid	Peak 1		Peak II	
	Molar ratio	Nearest integer	Molar ratio	Nearest integer
Asp	-	-	0.0643	0
Thr	0.0596	0	0.0272	0
Ser	-	-	0.0976	0
Glu	2.1500	1	0.1777	0
Gly	0.0638	0	0.1207	0
Ala	1.9731	1	0.0387	0
Cys	-	-	-	-
Val	1.6444	1	0.0441	0
Met	0.0561	0	0.0237	0
Ile	0.0501	0	0.0294	0
Leu	0.2031	0	2.0629	2
Tyr	0.0271	0	0.0161	0
Phe	1.9310	1	0.7983	1
His	0.0327	0	0.0360	0
Lys	0.1397	0	0.0497	0
Arg	0.1087	0	0.0528	0
Pro	4.2510	2	-	-
Suggested fragment Q-value	α_{s1}CN (f1-23) 1810		β-CN (f193-209) 2497	

4. CONCLUSIONS

This study explains the important role of X-PDP in the debittering process of casein hydrolysates. The incubation of bitter EMC with cell free extract from *Lactobacillus casei* ssp. *casei* LLG has resulted in the debittering of EMC within a few hours. Strong evidence has been found to relate the debittering effect of crude extract to the action of X-PDP.

REFERENCES

1. Peterson, S. D. & Marshall, R. T. (1990). Non starter lactobacilli in Cheddar cheese: A Review. *J Dairy Sci. 73*, 1395-1410.
2. Olson, N. F. (1990). The impact of lactic acid bacteria on cheese flavor. *FEMS Microbiol. Reviews. 87*, 131-147.
3. Richardson, B. C. & Creamer, L. K. (1973). Casein proteolysis and bitter peptides in Cheddar cheese. *N. Z. J. Dairy Technol. 8*, 46-51.
4. Schmidt, R. H. (1990). *Bitter compounds in dairy products*. Pp. 183-204. *In* Bitterness in food and beverages. Edited by R. L. Rousseff, Amsterdam, Elsevier.
5. Umetsu, H., Matsuoka, H. & Ichishima, E. (1983). Debittering mechanisms of bitter peptides from milk casein by wheat carboxypeptidase. *J. Agric. Food Chem. 31*, 50-53.
6. Minagawa, E., Kaminogawa, S., Tsukasaki, F. & Yamauchi, K. (1989). Debittering mechanism in bitter peptides of enzymatic hydrolysates from milk casein by aminopeptidase T. *J. Food Sci. 54*, 1225-1229.
7. Matsuoka, H., Fuke, Y., Kaminogawa, S. & Yamauchi, K. (1991). Purification and debittering effect of aminopeptidase II from *Penicillum caseicolum. J Agric. Food Chem. 39*, 1392-1397.
8. Tan, P. S. T., van Kessel, T. A. J. M., Van de Veerdonk, F. L. M., Zuerendonk, P. F. Bruins, A. P. & Konings, W. N. (1993). Degradation and debittering of a tryptic digest of β-casein by aminopeptidase N from *Lactococcus lactis* subsp. *cremoris* WG2. *Appl. Environ. Microbiol. 59*, 1430.
9. Baankers, R. (1992). In vitro breakdown of α_{s1}CN (f1-23) and β-CN (f193-209) by lactococcal peptidases. Ch 9.

www.SID.ir

In The role of lactococcal peptidases in cheese ripening. Ph. D thesis [University of Amsterdam.].

10. Leadbeater, L. & Ward, F. B. (1987). Analysis of tryptic digest of bovine β-casein by reversed phase high performance liquid chromatography. *J. Chromatogr. 397*, 435-443.

11. Habibi Najafi, M. B. & Lee, B. H. (1996). Bitterness in Cheese: A Review. *Crit. Rev Food Sci Nutr. 36*(5), 397-411.

12. Cliffe, A. J. & Law, B. A. (1990). Peptide composition of enzyme-treated Cheddar cheese slurries, determined by reversed-phase high performance liquid chromatography. *Food Chem. 36*, 73-80.

13. Law, B. A. & Wigmore, A. S. (1982). Accelerated cheese ripening with food grade proteinase. *J. Dairy Res. 49*, 137-146.

14. Park, S. Y., Gibbs, B. F. & Lee, B. H. (1995). Effect of crude enzyme of *Lactobacillus casei* ssp. *casei* LLG on water-soluble peptides of enzyme modified cheese. *Food Res. Int. 28*, 43-49.

15. Habibi Najafi, M. B. & Lee, B. H. (1994). Purification and characterization of x-prolyl dipeptidyl peptidase from *Lactobacillus casei* ssp. *casei* LLG. *Appl. Microbiol. Biotechnol. 42*, 280-286.

16. Park, S. Y., Arora, G. & Lee, B. H. (1993). Characterization of enzyme modified cheese flavor/aroma compounds. *J. Dairy Sci.* Suppl. *1*, 76,145.

17. Spackman, D. H., Stein, W. H. & Moore, S. (1958). Automated recording apparatus for use in the chromatography of amino acids. *Anal. Chem. 30*, 1190.

18. Veeragavan, K., Colpitts, T. & Gibbs, B. F. (1990). Purification and characterization of two distinct lipases from *Geotricum candidum. Biochem. Biophys. Acta*, *1044*, 26-33.

19. Covey, T. R., Huang, E. C. & Henion, J. D. (1991). Structural characterization of protein tryptic peptides via liquid chromatography/mass spectrometry and collision-induced dissociation of there doubly charged molecular ions. *Anal. Chem. 63*, 1193.

20. Holt, H. (1988). *Dairy products judging*. Publ 731, Agricultural External Services. Institute of Agriculture, University of Tennesse, USA: Knoxville.

21. Carles, C. & Ribadueau-Dumas, B. (1986). Determination of gradient elution conditions for the separation of peptide mixtures by reversed-phase high performance liquid chromatography: bovine β-casein tryptic digest. *J Dairy Res. 53*, 595-600.

22. Ney, K. H. (1979). Bitterness of peptides: Amino acid composition and chain length. *In* Food Taste Chemistry. Edited by J. C. Bondreau. ACS symposium series, *115*, 149-173.

23. Hamilton, J. S., Hil, R. D. & Van Leuween, H. (1974). A bitter peptides from Cheddar cheese. *Agric. Biol. Chem. 38*, 375-379.

24. Hodges, R., Kent, S. B. H & Richardson, R. C. (1972). The mass spectra of some permethylated acetylpeptides. *Biochem. Biophys. Acta. 257*, 54-58.

25. Pélissier, J. P., Mercier, T. C. & Ribadeau-Dumas, B. (1974). Etude de la proteolyse de caseines α$_{s1}$et β bovines par la presure. *Ann. Biol. Anim. Biochem. Biophys. 14*, 343-362.

26. Smid, E. J., Poolman, B. & Konings, W. N. (1991). Casein utilization by Lactococci. *Appl. Environ. Microbiol.* 57, 2447-2452.

27. Lee, B. H., Laleye, L. C., Simard, R. E., Holley, R. A., Emmons, D. B. & Grioux, R. N. (1990). Influence of homofermentative lactobacilli cultures on physico-chemical and sensory properties of Cheddar cheese. *J Food Sci. 55*, 386-390.

28. Lee, B. H., Laleye, L. C., Simard, R. E., Munsh, M. H. & Holley, R. A. (1990). Influence of homofermentative lactobacilli on the microflora and soluble nitrogen components in Cheddar cheese. *J Food Sci. 55*, 391-397.

29. Ishibashi, N., Ono, I., Kato, K., Shigenega, T., Shinoda, I., Okai, H. & Fukui, S. (1988 a). Role of the hydrophobic amino acid residue in the bitterness of peptides. *Agric. Biol. Chem. 52*, 91-94.

30. Ishibashi, N., Kubo, T., Chino, M., Fukui, H., Shinoda, I., Kikuchi, E., Okai, H. & Fukui, S. (1988 b). Taste of proline-containing peptides. *Agric. Biol. Chem. 52*, 95-99.