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Original article

Milk fermentation by *Lactococcus lactis* with modified proteolytic systems to accumulate potentially bio-active peptides

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Abstract – The proteolytic system of lactic acid bacteria has been characterised in detail and numerous modified strains with null or increased specific proteolytic activities have been constructed or identified among natural strains. Based on this knowledge, our objective was to ferment milk with modified strains and produce mixtures of peptides with specific features corresponding to potential bio-activities. We used a collection of *Lactococcus lactis* negative mutants for peptidase activities available in the laboratory to ferment the milk. In particular, we focused our work on mutants lacking either aminopeptidase N, X-prolyl dipeptidyl aminopeptidase or tripeptidase in order to test their ability to form peptides with immunomodulating or anti-hypertensive activities. At the end of fermentation, supernatants were fractionated by RP-HPLC. Each fraction collected was analysed by Maldi-Tof MS and sequencing. We observed that mutants accumulated specific peptides consistent with the specificity of the missing peptidases. Some of the peptides identified present similarities with peptides having immunomodulating or anti-hypertensive effects. One of these was quantified. At the same time, we observed that the inhibition of angiotensin converting enzyme was stronger in supernatants obtained with mutant strains than in supernatant obtained using the wild-type strain. In conclusion, we showed that in some cases, modifications to the proteolytic system of *Lactococcus lactis* gave rise to significant differences in the mixtures of peptides produced during milk fermentation. The differences in bio-activity of these peptide mixtures were only partially determined in vitro and evidently need to be demonstrated in vivo. Exploitation of the biodiversity of the proteolytic system of lactic acid bacteria may enable a direct application of this work and undoubtedly a promising means of directly producing natural bio-active peptides in fermented milk products.

Milk fermentation / *Lactococcus lactis* / bio-active peptide / proteolysis / mutant

Résumé – Conséquences de modifications du système protéolytique de *Lactococcus lactis* sur l'accumulation de peptides potentiellement bio-actifs pendant la fermentation du lait. Le système protéolytique des bactéries lactiques a été caractérisé en détail et de nombreuses souches aux activités protéolytiques modifiées ont été construites ou identifiées parmi des souches sauvages. Notre objectif est de produire des mélanges de peptides ayant des activités biologiques, en utilisant ces souches modifiées pour fermenter le lait. Pour cela, nous avons utilisé une collection de mutants de *Lactococcus lactis* dont certaines activités peptidasiques ont été supprimées. En particulier, nous nous sommes intéressés à des souches n'ayant plus l'aminopeptidase N, la X-prolyl-dipeptidyl aminopeptidase, ou la tripeptidase pour tester leur aptitude à former des peptides ayant des activités

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immuno-modulatrices ou anti-hypertensives. À la fin de la fermentation, les surnageants ont été fractionnés par RP-HPLC. Les fractions collectées ont alors été analysées par spectrométrie de masse (Maldi-Tof) et séquençage. Nous avons pu constater que les mutants accumulent des peptides, dont la séquence est en accord avec la spécificité de la peptidase manquante. Certains des peptides identifiés ont des séquences proches de peptides connus comme ayant une activité immunomodulatrice ou anti-hypertensive. L'un d'eux a été quantifié. Parallèlement, nous avons observé une augmentation de l'inhibition de l'enzyme de conversion de l'angiotensine dans les surnageants obtenus à partir des mutants par comparaison au surnageant obtenu avec la souche sauvage. En conclusion, nous avons montré que les modifications du système protéolytique de *Lactococcus lactis* induisent des différences notables au niveau des peptides produits pendant la fermentation. Les différences d'activité biologique observées in vitro demandent bien entendu à être démontrées in vivo. L'exploitation de la biodiversité du système protéolytique des bactéries lactiques est une application directe de ce travail et sans aucun doute une porte ouverte sur la production naturelle de peptides bioactifs dans les produits laitiers fermentés.

Lait fermenté / *Lactococcus lactis* / peptide bioactif / protéolyse / mutant

1. INTRODUCTION

At present, milk proteins are the principal known source of bio-active peptides [2]. To exert their physiological effects, bioactive peptides must be released by proteolysis and then attain their target sites in the lumen of the intestinal tract or, after absorption, in peripheral organs. In fact, bio-active peptides are released either during the in vivo gastrointestinal digestion of appropriate precursors or during food processing by endogenous or microbial enzymes. The well-established bio-activities recently reviewed by Meisel [11] concern the treatment of diarrhoea, hypertension, thrombosis, dental disease, immunodeficiency and an increase in mineral absorption. The relationship between peptide structures and bio-activities has not been elucidated in all cases, even though it has been shown that peptides exhibiting the same activity usually share the same structural features. One of the simplest ways of producing bio-active peptides is to use lactic acid bacteria, which are generally recognised as safe and are traditionally used to ferment milk. In this manner, bio-active peptides are directly released into a food, requiring no purification or processing. Compared with production in an enzymatic reactor or by chemical synthesis, this method is also less expensive. However, the production of bio-active peptides through milk fermentation by lactic acid bacteria remains poorly documented [10, 17, 21], and no clear relationship has been established between the probiotic character of some dairy bacteria and their ability to produce specific peptides. At present, two fermented milks, with a proven anti-hypertensive effect in humans, are on the market in Japan and Finland. Their beneficial effects are attributed to the two tripeptides, Ile-Pro-Pro and Val-Pro-Pro, which are released during fermentation [6, 14, 18]. Apart from these examples, few studies have demonstrated the bio-activity of fermented products in vitro. However, the presence of peptides known to be bio-active has been reported in several dairy products. But despite detailed knowledge of the proteolytic system of lactic acid bacteria [1], the potential for these bacteria to release bio-active peptides is still unpredictable and poorly controlled. In a previous work, using isogenic *Lactococcus lactis* strains differing in terms of their peptidase contents, we demonstrated that the quantities of amino acids released during cheese-ripening varies, depending on the peptidases not present [5]. Therefore, we could also anticipate changes to the peptide content of fermented milk with these *Lactococcus lactis* modified strains. The objective of the present study was to confirm these differences in milk proteolysis and to assess the possible consequences of changes observed

in the bio-activity of fermented milks. Special attention was paid, firstly, to the general aminopeptidase PepN, the proline-specific X-prolyl dipeptidyl aminopeptidase PepX, and the tripeptidase PepT, and secondly, to anti-hypertensive and immunomodulating peptides.

2. MATERIALS AND METHODS

2.1. Strains

Five *Lactococcus lactis* ssp. *cremoris* strains differing in terms of their proteolytic systems were compared: wild-type strain TIL46 (NCDO763) and its derivatives in which the *pepN*, *pepT* and *pepX* genes encoding the general aminopeptidase PepN, the tripeptidase PepT and the X-prolyl dipeptidyl aminopeptidase PepX had been inactivated [5].

2.2. Cultures

Strains were grown in M17 broth overnight at 30 °C. This culture was used to inoculate a gallery (4, 2, 0.5, and 0.25%, v/v) of skimmed milk (Nilac, NIZO, Ede, The Netherlands). After 8 h at 30 °C, cultures exhibiting the best growth (but which had not clotted) were chosen to inoculate 2% (v/v) skimmed milk (Nilac). The cultures were incubated at 30 °C for 16 h (clotted milk). The supernatants were recovered by centrifugation (5000 × *g*, 4 °C, 15 min).

2.3. HPLC

Supernatants (diluted in solvent A (50/50), and if necessary, centrifuged again) were analysed by RP-HPLC on a Hypersil 100 C_{18} column (Thermo-Finnigan, Courtabœuf, France), kept at 40 °C, with a flow-rate of 1 mL·min^{-1} and detection at 220 nm. The following two-solvent system was used: solvent A: 0.115% TFA in water; and solvent B: 0.1% TFA in 100% CH_3CN. The following gradient was applied: 10 min of solvent A followed by a gradient from 0 to 60% of solvent B in 50 min. In order to determine the principal peaks, another gradient with a slower slope was employed: solvent A for 5 min, then from 0 to 48% of solvent B in 80 min. Fractions were collected every 30 s.

In order to analyse specifically those peptides smaller than 500 g·mol^{-1}, supernatants were ultrafiltered on YC05 membranes using the MPS-1 micropartition system (Amicon Division, Danvers, MA, USA).

2.4. Mass spectrometry

Supernatants and purified fractions were analysed using a MALDI-TOF mass spectrometer (Voyager DE-STR; Applied Biosystems, Palo-Alto, CA, USA) with a laser at 337 nm and an acceleration voltage of 20.000 V. Samples were prepared as follows: 1 µL of a sample was applied to the sample plate. When the sample was dry, 1 µL of an α-CHCA matrix (5 mg·mL^{-1} α-cyano-4-hydroxycinnamic acid in 50:50 CH3CN: TFA, 0.3%, v/v) was added.

2.5. Peptide sequencing

Peptide sequences were obtained using the Edman method with an automatic protein sequencer (494A protein sequencer, Applied Biosystems).

2.6. Angiotensin converting enzyme (ACE) inhibitory in vitro test

The inhibition of ACE was measured according to the method described by Holmquist et al. [7], adapted to the screening of bio-active peptides [20]. FAPGG (Furylacryloyl-Phe-Gly-Gly, ACE reagent, Sigma Aldrich, St Louis, MO, USA) was hydrolysed to FAP and GG through the action of ACE (ACE from pig kidney, Sigma). The hydrolysis of FAPGG results in a reduction in absorbance at 340 nm. ACE activity in the sample was determined by comparing the sample reaction rate with that obtained using a control, the latter corresponding to the maximum activity of ACE with no inhibitor.

Table I. Peptides identified in the supernatant of milk fermented with *Lactococcus lactis* wild-type strain after separation by RP-HPLC. Cultures on skimmed milk (Nilac) incubated at 30 °C for 16 h. Analysis by RP-HPLC on a Hypersil 100 C_{18} column, 1 mL·min^{-1}, at 220 nm. Solvent A (0.115% TFA in water) for 5 min, then from 0 to 48% of solvent B (0.1% TFA in 100% CH_3CN) in 80 min.

Peptide	Protein	Position	Peptide	Protein	Position
GKEKVNE	αs_1-CN	33-39	SITRIN	β–CN	22-27
DIKQM	αs_1-CN	56-60	SITRINK	β–CN	22-28
AESIS	αs_1-CN	62-66	KIEK	β–CN	29-32
SVEQ	αs_1-CN	75-78	KEIKF	β–CN	29-33
SVEQKHIQK	αs_1-CN	75-83	EQQQTED	β–CN	37-43
DVPSER	αs_1-CN	85-90	EQQQTEDEL	β–CN	37-45
YKVPQLE	αs_1-CN	104-110	QQQTEDE	β–CN	38-44
SMKEG	αs_1-CN	122-126	AQTQSLVYPF	β–CN	37-45
DAYP	αs_1-CN	157-160	SLPQNPPLT	β–CN	38-44
			GVSKVKEAMA	β–CN	94-103
SEESI	αs_2-CN	10-14	KVKEAMAPKH	β–CN	97-106
SIISQEIYK	αs_2-CN	13-21	KHKEM	β–CN	105-109
SEESAEV	αs_2-CN	58-64	EMPF	β–CN	108-111
EVATE	αs_2-CN	63-67	KYPV	β–CN	113-116
FYQKFPQ	αs_2-CN	88-94	TESQSL	β–CN	120-125
KFPQYL	αs_2-CN	91-96	VPQKA	β–CN	173-177
YQGP	αs_2-CN	100-103	KAVPY	β–CN	176-180
AVIP	αs_2-CN	116-119	KAVPYPQ	β–CN	176-182
ALPQYL	αs_2-CN	175-180	VPYP	β–CN	178-181
KVIPY	αs_2-CN	199-203	YPQR	β–CN	180-182
			YQEPVLGPVRGPFPIIN	β–CN	193-209
KYIP	κ–CN	24-27			
YAKPA	κ–CN	61-65	YAPE	BSA	149-152
SPAQIL	κ–CN	69-74			
TIASGEPTSTPTT	κ–CN	124-136			
VIESPP	κ–CN	152-157			
VIESPPEIN	κ–CN	152-160			
SPPEI	κ–CN	155-159			
TVQVTSTAV	κ–CN	161-169			
STAV	κ–CN	166-169			

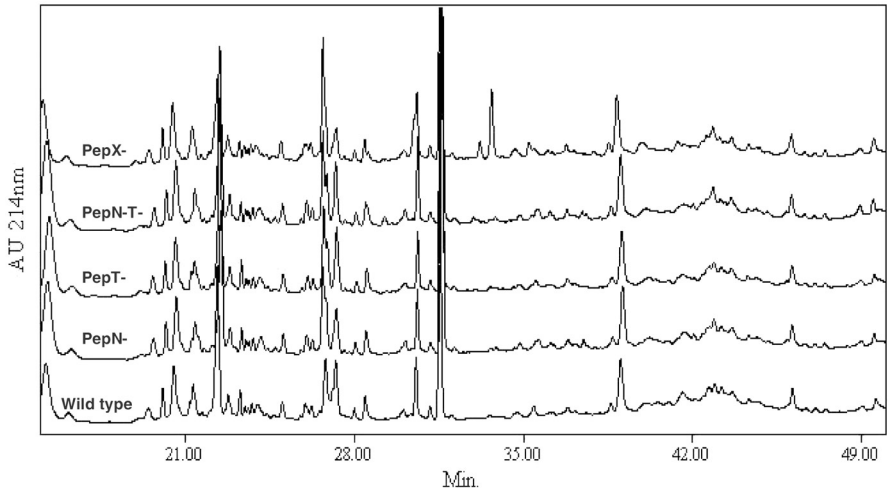

Figure 1. RP-HPLC chromatograms of the supernatant of milk fermented with the *L. lactis* wild-type strain and peptidase negative mutants. Analysis by RP-HPLC on a Hypersil 100 C_{18} column, 1 mL·min^{-1}, at 220 nm. 10 min with solvent A (0.115% TFA in water) followed by a gradient from 0 to 60% of solvent B (0.1% TFA in 100% CH_3CN) in 50 min.

3. RESULTS AND DISCUSSION

3.1. Peptide content of fermented milks with peptidase mutants of *Lactococcus lactis*

The peptide contents of fermented milk supernatants were analysed using RP-HPLC and mass spectrometry. Forty-eight peptides present in the supernatant of the wild-type strain were identified. They originated from the four main caseins and serum albumin and ranged in size from 4 to 17 amino acids (Tab. I). The sequences of most of these peptides did not match with the sequences of peptides released during the incubation of purified cell wall proteinase from the wild-type strain with purified caseins [12, 13]. This observation suggests that either the peptides present in the supernatant did not derive from casein hydrolysis by cell wall proteinase alone, or that cell wall proteinase did not exhibit the same specificities in cell-bound and free forms, as has already been demonstrated for other *L. lactis* strains [4]. The peptides identified in the supernatant of the wild-type strain have a high acidic amino acid content in line with the specificity of the *L. lactis* transport system which preferentially transports basic peptides [8].

Comparison of the RP-HPLC chromatograms revealed that only that obtained with the PepX *L. lactis* mutant significantly differed from the others; those obtained with the PepN, PepT and PepN-PepT mutants were similar to that obtained with the wild-type strain (Fig. 1). Two main differences between the wild-type strain and PepX mutant chromatograms were identified and corresponded to an accumulation of two peptides. Their sequences, APFPE (α_{s1}-CN 26-30) and VPYPQ (β-CN 178-182), were in agreement with the specificity of PepX, which is the sole lactococcal peptidase which efficiently releases X-Pro dipeptides from the N-termini of peptides. The amount of VPYPQ peptide accumulating in the supernatant of the PepX mutant was estimated (from amino acid analysis) to be 120 µg·L^{-1}. Analysis of a milk fermented

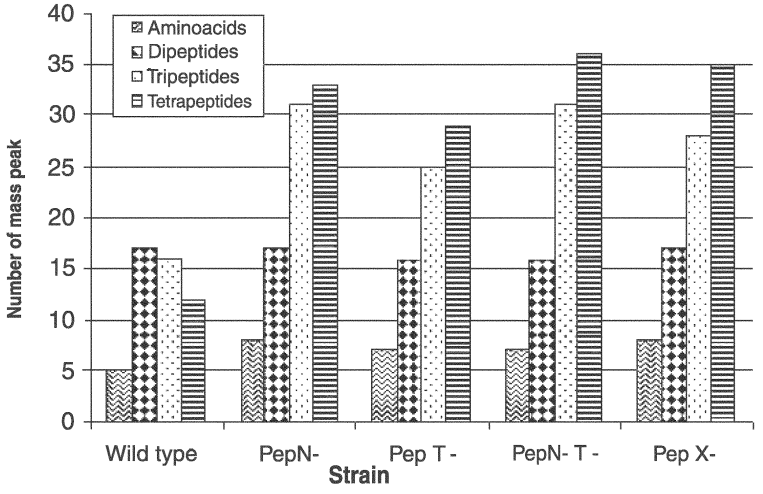

Figure 2. Distribution of mass peaks of the supernatants of milk fermented with the *L. lactis* wild-type strain and peptidase negative mutants (for molecules with masses <700 g·mol^{-1}).
This figure is provided for information, based on a single analysis with a Maldi-Tof spectrometer and corresponding to the number of mass peaks observed for the interval of mass for amino acids (75-205), dipeptides (130-390), tripeptides (190-580), and tetrapeptides (250-760). In this case, the matrix used was THAP (2,4,6-trihydroxyacetophenone).

with a PepX mutant of *Lactobacillus helveticus* revealed an accumulation of β-casomorphin 1-4 (YPFP), another peptide comprising an alternation of prolyl and other residues [9].

Because all peptides are not easily detectable by UV, and because some of them were not retained on the RP-HPLC column because of their low hydrophobicity and their small size, we also analysed fractions of milk supernatants containing peptides smaller than 500 g·mol^{-1} by mass spectrometry. We compared the distribution of mass peaks between strains (Fig. 2). All supernatants obtained with mutants exhibited about 50% more peaks with masses corresponding to tri- and tetra-peptides, suggesting that because of a proteolysis defect in these strains, peptide hydrolysis was incomplete, leading to an accumulation of small peptides. PepN exhibited the strongest affinity for peptides with three to six residues [16], and PepT was the only lactococcal peptidase to specifically hydrolyse tripeptides. We expected that inactivation of their genes would lead to an accumulation of short peptides. This hypothesis was in line with the additional mass peaks observed in mutant supernatants within the range of tri- and tetra-peptide masses. Because the strains used only differed in terms of their peptidase content, we can reasonably speculate that the additional mass peaks corresponded to additional peptides in mutant supernatants.

3.2. Potential bio-activities of supernatants

We employed an in vitro ACE assay to evaluate the inhibition activity of different supernatants. ACE activity was reduced by 50% in the presence of the wild-type strain supernatant, and by more than 70% with the supernatants of the four mutants. These results confirmed the increased ACE inhibitory activity of milk by bacterial fermentation [19] and revealed the significant beneficial

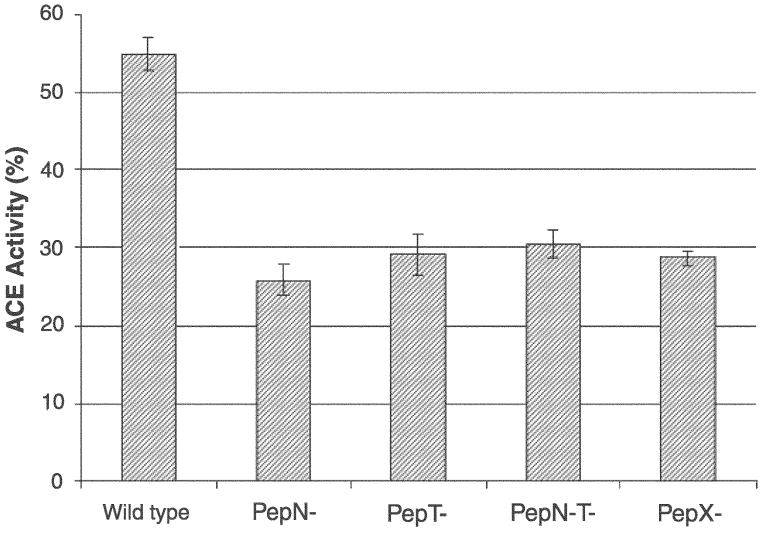

Figure 3. Inhibition of angiotensin converting enzyme (ACE) by the supernatants of milk fermented with the *L. lactis* wild-type strain and peptidase negative derivatives. The results are given as a percentage of the control value (= 100) corresponding to the maximum activity of ACE when the supernatant was replaced by water.

effect of peptidase gene inactivation on the inhibitory activity of supernatants (Fig. 3). Use of this in vitro assay was a preliminary approach and inevitably raised the question of its in vivo validation. Undoubtedly, the level of correlation between in vitro and in vivo ACE inhibition assays requires extensive evaluation. However, at least in the case of fermented milks containing the IPP and VPP tripeptides, an in vitro inhibitory activity has been confirmed in vivo by experiments in rats and humans [6, 15, 19].

The bio-activity of peptides accumulating in the supernatant of the PepX negative strain has not yet been measured. However, peptides specific to this strain belong to the interesting group of peptides comprising an alternation of prolyl and other amino acid residues. These peptides are generally endowed with one or several biological activities, including ACE inhibition, immunomodulation and opioid activity, as summarised in Table II [3, 11]. An accumulation of such peptides is also interesting insofar as their high proline content suggests they may be more resistant than other peptides to further proteolysis.

4. CONCLUSIONS AND PERSPECTIVES

This study demonstrated that the composition of the proteolytic system of lactic acid bacteria influenced the peptide content of milk at the end of fermentation and, specifically, as a function of the missing peptidases. Consequently, such modifications can modulate the bio-activity of fermented milks, at least in vitro. This observation opens the way to using lactic acid bacteria (selected for their proteolytic properties and capable of producing specific peptides at a low cost) directly in a dairy product. This work, in which we used genetically-modified organisms, can now be extended to the search, among lactic acid bacteria, of naturally-existing negative mutants for peptidase

Table II. Peptides from the literature comprising a repetition of the X-Pro pattern, and their reported bio-activities. [1] IC_{50} values ($\mu mol \cdot L^{-1}$) are given for peptide concentrations inhibiting [^{3}H]-ligand binding by 50%. [2] IC_{50} values ($\mu mol \cdot L^{-1}$) are given for peptide concentrations inhibiting the activity of angiotensin converting enzyme (ACE) by 50%. [3] Figures indicate the maximum % stimulation (+) and/or inhibition (–), respectively, by comparison with controls (= 100).

Peptide sequence	Fragment	Biological activity		
		Opioid	ACE inh.	Immunomodulator [3]
		IC_{50}[1]	IC_{50}[2]	
AVPYP[4]	β 177-181		80	
AVPYPQR[4,5]	β 177-183		15	
YPFPG[5]	β 60-64	1.1		
YPFPGPI[4,5]	β 60-66	14	500	– 21/+ 26
YPFPGPIPNSL[5]	β 60-70	10		
PGPIPN[5]	β 63-68			+ 139
EMPFPK[4]	β 108-113		423	
KVLPVP[4]	β 169-174		5	
KVLPVPQ[4]	β 169-175		>1000	

[4] From Fitzgerald and Meisel [3]; [5] from Meisel and Bockelmann [11].

activities (P. Tailliez, personal communication). Numerous experiments are now necessary to study in vivo the behaviour of these peptides of interest, i.e. after ingestion in animals and then in humans, in order to determine how they resist proteolysis and if they can reach the blood or specific receptors.

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