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**Note**

# Growth-promoting activity of tryptic digest of caseinomacropeptide for *Lactococcus lactis* subsp *lactis*

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**Summary** — Tryptic digest of caseinomacropeptide (C-terminal part of κ-casein) was separated into 2 fractions by ultrafiltration membrane (cut-off point 3 000 Da). Both fractions strongly stimulated, in a dose–response-dependent manner, *Lactococcus lactis* subsp *lactis* CNRZ 1076 growth in reconstituted skim milk. Growth rates were 2-fold higher than that observed in the control culture.

**caseinomacropeptide / hydrolysis / stimulation / *Lactococcus lactis***

**Résumé — Stimulation de la croissance de *Lactococcus lactis* subsp *lactis* par l'hydrolysat trypsique du caséinomacropeptide.** *L'hydrolysat trypsique du caséinomacropeptide (fraction C-terminale de la caséine κ) a été séparé en 2 fractions par ultrafiltration sur membrane ayant un seuil de coupure de 3 000 Da. Ces 2 fractions stimulent fortement la croissance de* Lactococcus lactis *subsp* lactis *CNRZ 1076 en milieu lait écrémé selon un effet dont l'intensité dépend de la dose utilisée. Le taux de croissance cellulaire est multiplié par deux par rapport à celui observé sur le lait témoin.*

***caséinomacropeptide / hydrolyse / stimulation /* Lactococcus lactis**

## INTRODUCTION

Caseinomacropeptide (CMP), the glycosylated C-terminal portion (64 amino-acids) of κ-casein, is released by rennet action during milk clotting. Tryptic hydrolysis of this fragment leads to the liberation of small peptides (SP) (4–7 amino acids) from the N-terminal part (Léonil and Mollé, 1990). These molecules constitute a family of bioactive peptides with antithrombotic activity (Jollès *et al,* 1986; Maubois *et al,* 1991). The high molecular weight difference between these bioactive peptides ($M_r$ < 700 Da) and complementary fragments ($M_r$ > 6 000 Da) was exploited for continuous separation of SP in the membrane reactor (Bouhallab *et al,* 1992).

Moreover, SP-enriched permeate could be a potential nutriment for lactic acid bacteria because it has been reported that peptides of 4–6 residues and less are favourable for microorganism growth (Desmazeaud and Hermier, 1973; Law *et al*, 1976).

The purpose of this work was to study the effect of separated tryptic digest of CMP on *Lactococcus lactis* subsp *lactis* CNRZ 1076 growth in skim milk media. Cell viability determination and acidification rate of milk were used to assess growth-promoting activity of these molecules.

## MATERIALS AND METHODS

Substrate, caseinomacropeptide, was prepared according to Brulé *et al* (1980). Trypsin (EC 3.4.21.4) was from Novo Nordisk Bioindustrie SA (Fontenay-sous-Bois, France). *Lactococcus lactis* subsp *lactis* CNRZ 1076 was used as assay organism.

### *Tryptic hydrolysis of CMP*

Ten g of CMP were incubated with trypsin (E/S molar ratio = 1:210) in 650 ml distilled water at pH 8 and 40 °C. The pH was controlled by continuous addition of 0.1 N NaOH using pH-stat (Metrohm, Roucaire, France). After 3 h, the hydrolysate was concentrated to 125 ml using a Filtron minisette system (Pharmacia, Saint-Quentin-en-Yvelines, France) equipped with an ultrafiltration membrane of 3 000 Da cut-off. The permeate (500 ml) enclosing SP was freeze-dried. The retentate (LP fraction) was diafiltered with 2 vol distilled water and then extensively dialysed by using Spectra-por 1000 Da cut-off dialysis tubing. Amino acid composition was carried out according to Bidlingmeyer *et al* (1984).

### *Culture conditions and growth media*

*Lactococcus lactis* cells were stored in M17 medium with 15% (w/w) glycerol added at –20 °C. Pre-culture and cells preparation were carried out in skim milk according to Juillard and Richard (1989). For growth experiments, exponentially growing cells were used to inoculate reconstituted skim milk (Lait G, ITG, La Roche sur Foron, France) at $10^4$ CFU/ml. Incubation temperature was 30 °C.

### *Acidification rate and cells viability*

Acidification rate of culture medium by bacteria was monitored using multi-pH meter (Solomat, Evry, France). Bacterial enumeration was carried out by plating sample dilutions on M17 medium with a Spiral plater (Interscience, St Nom-la-Bretèche, France). Plates were incubated for 24 h at 30 °C before reading. Growth rate ($\mu$) was calculated by linear regression using Monod's equation (Monod, 1958).

### *Activity tests*

Potential activities of peptide solutions on acid production were determined by addition of 1 ml of each adequate dilution to 19 ml culture medium. The effects of peptide on bacterial growth were determined by addition of 5 ml of peptide solution to 95 ml culture medium. For control cultures, water was added instead of peptide solutions.

## RESULTS AND DISCUSSION

By using ultrafiltration membrane separation technique, tryptic hydrolysate of CMP was separated into 2 groups of peptides: SP ($M_r$ 500–700 Da) and LP (average $M_r$ 6 000 Da) (Bouhallab *et al*, 1992). Both fractions stimulate *Lactococcus lactis* growth in reconstituted skim milk. As shown in figure 1, they strongly promote the pH decrease of the culture medium in a dose–response-dependent manner. The minimal concentration of SP required for detecting promoting activity under our conditions was 0.2 $\mu$g/ml. The growth promot-

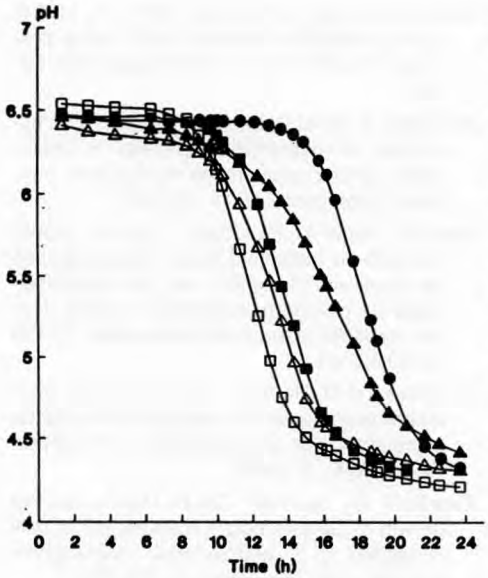


**Fig 1.** Acid production activity of *L lactis* (—●—) control; (—□—) SP 50 µg/ml; (—■—) SP 5 µg/ml; (—Δ—) LP 2 mg/ml; (—▲—) 0.5 mg/ml. Growth experiments were carried out in 20 ml reconstituted skim milk.
*Activité acidifiante de* L lactis *(—●—) témoin; (—□—) (—■—) petits peptides à 50 et 5 µg/ml respectivement; (—Δ—) (—▲—) gros peptides à 2 et 0,5 mg/ml respectivement. La croissance était réalisée dans 20 ml de lait écrémé reconstitué.*

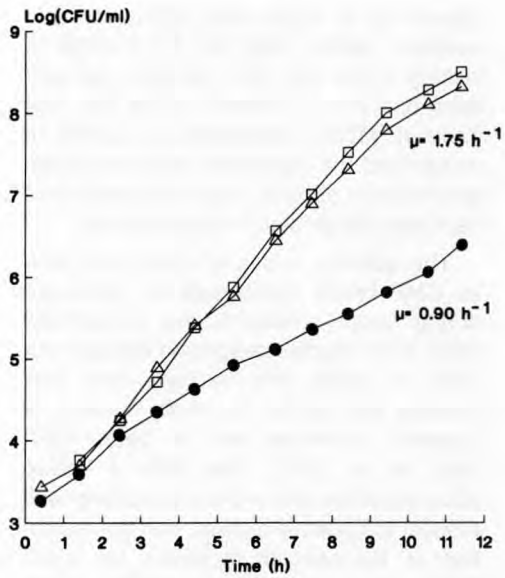


**Fig 2.** Effects of SP (50 µg/ml) (—□—) and LP (2 mg/ml) (—Δ—) on *L lactis* growth as determined by cells enumeration on M17 medium (—●—) control culture. µ = growth rate.
*Effets des petits peptides (50 µg/ml) (—□—) et des gros peptides (2 mg/ml) (—Δ—) dérivés du CMP sur la viabilité du* L lactis. *(—●—) Témoin. µ = taux de croissance.*

ing-property of CMP-derived peptides was confirmed by enumeration of viable cells (fig 2). Growth rates were 2-fold higher when peptidic fractions were added than that of control culture. These observed activities of CMP derived fractions were much higher than that obtained by addition of whole CMP in the same conditions (unpublished results).

Mechanisms through which CMP-derived peptides induce *Lactococcus lactis* growth are probably different. The fractions tested differ by their average $M_r$ as well as in amino acid composition (table I). They

**Table I.** Amino acid composition of the fractions derived from CMP.
*Composition en acides aminés des 2 fractions obtenues par hydrolyse trypsique du CMP.*

| *SP* | *LP* |
|---|---|
| Ala[1a]; Asn[1]; Asp[1] Gln[1]; Ile[1b]; Lys[3] Met[1b]; Pro[2] | Ala[5/4*]; Asn[2]; Gln[1]; Glu[8b]; Gly[1]; Ile[6/5b*]; Leu[1b]; Pro[6]; Ser[6]; Thr[11/12*]; Val[6b]; Asp[1*] |

[a] Mol amino acid/mol CMP; [b] essential amino acids according to Marshall and Law (1983); SP: small peptides; LP: large peptides; * from sequence data of CMP variants B/A, Grosclaude *et al* (1972).

cannot act as simple essential amino acid suppliers seeing that the SP fraction is lacking in the His, Glu, Leu and Val residues and the LP fraction in His and Met (table I). More investigation is needed to understand the absorption and stimulation mechanisms of both fractions as well as of each peptide present in both mixtures.

The question arises whether such similar CMP tryptic hydrolysate is produced during usual cheesemaking technology. Most of the studies describing nitrogen nutrition of lactic acid bacteria from milk proteins are related to whole caseins or β-casein (Exterkate and de Veer, 1987; Smid *et al,* 1991). Very little is known about peptides derived from κ-casein with respect to bacterial growth, with the exception of *Bifidobacterium* genus for which Poch and Bezkorovainy (1991) have reported that κ-casein was the main microbial growth promoter and that the active component was located in the *para* κ-casein part. No activity of the carbohydrate moiety was detected in this latter study in contrast to what had been previously described by Azuma *et al* (1984). It appears, nevertheless, that CMP can be split by cell wall located proteinase of *Lactococcus lactis* but is a poor substrate for the partially purified enzyme (Monnet *et al,* 1992).

However, the high promoting growth activity of CMP-derived peptides the smallest of which ones are known as possessing antithrombotic activity emphasizes the interest in investigating the mechanisms inducing the phenomenon described in this study.

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