JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Variability of Hydrolysis of β-, α_{s1}-, and α_{s2}-Caseins by 10 Strains of *Streptococcus thermophilus* and Resulting Bioactive Peptides

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ABSTRACT: Milk proteins contain numerous potential bioactive peptides, which may be released by digestive proteases or by the proteolytic system of lactic acid bacteria during food processing. The capacity of *Streptococcus thermophilus* to generate peptides, especially bioactive peptides, from bovine caseins was investigated. Strains expressing various levels of the cell envelope proteinase, PrtS, were incubated with α_{s1}-, α_{s2}-, or β-casein. Analysis of the supernatants by LC-ESI-MS/MS showed that the β-casein was preferentially hydrolyzed, followed by α_{s2}-casein and then α_{s1}-casein. Numbers and types of peptides released were strain-dependent. Hydrolysis appeared to be linked with the accessibility of different casein regions by protease. Analysis of bonds hydrolyzed in the region 1−23 of α_{s1}-casein suggests that PrtS is at least in part responsible for the peptide production. Finally, among the generated peptides, 13 peptides from β-casein, 5 from α_{s2}-casein, and 2 from α_{s1}-casein have been reported as bioactive, 15 of them being angiotensin-converting enzyme inhibitors.

KEYWORDS: *Streptococcus thermophilus, bioactive peptides, cell envelope proteinase, casein hydrolysis*

INTRODUCTION

In recent years, a link between the prevalence of certain diseases commonly encountered in Western countries, such as cardiovascular diseases, obesity, diabetes, cancer, and dietary factors, has been emphasized. The engineering and manufacturing of functional ingredients and foods is emerging to counter these pathologies. Among them, fermented dairy products have received much attention because they contain several compounds, such as bioactive peptides, obtained by milk protein hydrolysis.[1,2] Bioactive peptides are encrypted within many dietary protein sequences and, when released by various proteases, exercise physiological functions in the human body with a wide range of potential applications (heart, bone, dental, and digestive health; weight management; immunomodulation; mood swings, memory, and stress control).[3] Bioactive peptides described in the literature have been released from dietary proteins either by enzymes of the digestive tract such as trypsin or by the proteolytic system of microorganisms during food manufacture.[3,4] Numerous studies deal with the capacity of lactococci or lactobacilli to generate bioactive peptides from milk proteins during fermentation.[3] These studies were carried out on pure cultures of species such as *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis* subsp. *cremoris*, *Lb. acidophilus*, *Lb. casei*, *Lb. jensenii*, *Lb. reuteri*, *Lb. rhamnosus*, *Lc. lactis* ssp. *lactis*, *Lc. raffinolactis*, *Leuconostoc mesenteroides* ssp. *cremoris*, and numerous strains of *Lb. helveticus* or cocultures with industrial yogurt starters (*Lb. delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*).[5−9]

Although it belongs to the *Streptococcus* genus, *S. thermophilus* is classified as "Generally Recognized As Safe".[10] It is the second lactic acid bacterium (LAB), after *Lc. lactis*, in terms of industrial use.[11] *S. thermophilus*, having strong acidifying capacities, plays an important role in the initial stages of dairy product manufacture. To our knowledge, very few studies have proved the ability of this bacterium to produce bioactive peptides from milk proteins, perhaps because its proteolytic capacity was considered to be very weak. In particular, 16 peptides were characterized in a study on milk proteolysis by pure or mixed cultures of *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*.[12] Two of these peptides correspond to potential angiotensin-converting enzyme inhibitors.

The proteolytic system of *S. thermophilus* consists of a facultative cell envelope proteinase (CEP) named PrtS, an ABC-transporter of oligopeptides and di/tripeptide transporter, and a pool of intracellular peptidases.[13] It has long been believed that the high cellular density of *S. thermophilus* in milk depends upon its cocultivation with other bacteria such as *Lb. bulgaricus* during the manufacture of fermented dairy products;[14] with this assumption in mind, the screening of 97 strains of the collection of the Institut National de la Recherche Agronomique (INRA, France) showed that only 3 strains were PrtS$^+$ and probably able to grow at high density in milk because they were able to strongly acidify it during their growth.[15] However, recent publications report the presence of the CEP PrtS in numerous strains.[16−18] In a previous work, we clustered into 3 groups 30 strains of our laboratory collection on the basis of their ability to acidify milk during their growth: high (H)-, low (L)-, and medium (M)-acidifying strains.[16] We showed that all of the H-strains possess the *prtS* gene (*prtS*$^+$ genotype) and expressed it (PrtS$^+$ phenotype), whereas the L-strains were *prtS*$^-$. For the M-strains, the situation was more complex. Indeed, 62% had a *prtS*$^+$ genotype and displayed a

Received: June 1, 2011
Revised: November 20, 2011
Accepted: November 21, 2011
Published: November 21, 2011

PrtS$^-$ or PrtS$^\pm$ phenotype, this last phenotype corresponding to a very weak protease activity.

The aim of this work was to determine (i) whether *S. thermophilus* was able to generate bioactive peptides from purified bovine α_{s1}-, α_{s2}-, and β-casein (CN) as protein source through the activity of its proteolytic surface system; (ii) whether such a capacity could vary among the *S. thermophilus* species; (iii) whether the CEP PrtS could account for such a production, at least in part. Hence, the proteolytic capacities of six H-strains, four M-strains, and one L-strain (strain CNRZ1066, which is *prtS*$^-$) have been investigated.

MATERIALS AND METHODS

Bacterial Strains, Media, and Growth Conditions. *S. thermophilus* strains used in this work are listed in Table 1. They were isolated in our laboratory either from yogurt or from cheese (P. Bracquart, personal communication) or came from the Centre National de Recherches Zootechniques (CNRZ) (INRA, Jouy-en-Josas, France) or American Type Culture Collection (ATCC) (Manassas, VA) collections. The genome of the L-strain CNRZ1066 and that of the H-strain LMD-9 are sequenced.[19,20] All of the strains used in this work were *prtS*$^+$ (except strain CNRZ1066) but express PrtS at different levels. Strain CNRZ1066 was used to evaluate CN proteolysis in the absence of PrtS. Strains were stored at −80 °C in reconstituted skim milk (10%, w/v). *S. thermophilus* strains were inoculated at 1% in skim milk and incubated overnight at 42 °C before each experiment. Precultures were then inoculated at 1% in M17 medium[21] supplemented with lactose (20 g/L) and incubated at 42 °C until $OD_{650\ nm}$ reached 1.

Table 1. *S. thermophilus* Strains Used in This Work[a]

S. thermophilus strain	origin	strain type	*prtS*	PrtS
ATE19PB8	yogurt	H	+	+
Y4	yogurt	H	+	+
4F44	cheese	H	+	+
LMD-9*	yogurt	H	+	+
PB302	yogurt	H	+	+
PB385	yogurt	H	+	+
CNRZ404	yogurt	M	+	±
CNRZ445	cheese	M	+	±
ATCC19258	pasteurized milk	M	+	±
HAD8α	yogurt	M	+	−
CNRZ1066*	yogurt	L	−	−

[a]Key: *, genomes entirely sequenced; strain type H (high acidifying strain), L (low acidifying strain), and M (medium acidifying strain); genotype *prtS* (+, presence of the gene; −, absence). Phenotype PrtS is only qualitative (+, strong proteolytic activity; ±, weak proteolytic activity; −, no proteolytic activity) and was determined by zymography.[16].

Measurement of Intracellular Peptidase Activity. An intracellular peptidase activity, potentially released into the medium after cell lysis, was sought using the chromogenic substrate Lys-pNA. The substrate was prepared at a concentration of 2 mM, in 50 mM sodium phosphate buffer, pH 7.5. Each strain used in this work was grown in M17 medium supplemented with lactose (20 g/L) until $OD_{650\ nm}$ reached 1, and cells were harvested by centrifugation at 4000*g* for 5 min at 20 °C. The cell pellet was washed two times with 50 mM sodium phosphate buffer, pH 7.5, prewarmed at 42 °C, and resuspended at an $OD_{650\ nm}$ of 1 in the same buffer. The cells were then incubated at 42 °C, and an aliquot was collected immediately and then after 4 and 22 h. Cells were eliminated by centrifugation at 12000*g* during 5 min at 4 °C, and the supernatant was filtered through a 0.45 μm filter. One hundred microliters of the supernatant was then mixed with 1 mL of Lys-pNA solution and incubated for 2 h at 37 °C, and the absorbance was determined spectrophotometrically at 410 nm (Uvikon, Kontron, Switzerland). To ensure that intracellular peptidases of *S. thermophilus* were able to hydrolyze Lys-pNA substrate, intracellular proteins were extracted from the cell pellets, as previously described,[16] and incubated in the same conditions as above.

Casein Preparation. Bovine sodium caseinate was prepared and fractionated to obtain pure β-CN variant A2, according to the method of Girardet et al.,[22] and α_{s2}-CN variant A and α_{s1}-CN variant B, as previously described except that EDTA was not added to the CN fractions after their elution.[23]

Casein Hydrolysis. Cells were grown in M17 medium supplemented with 20 g/L lactose until $OD_{650\ nm}$ reached 1 and harvested by centrifugation at 4000*g* for 5 min at 20 °C. The cell pellet was washed in 50 mM sodium phosphate buffer, pH 7.5, prewarmed at 42 °C and was resuspended in 1.5 mL of the same buffer. Five hundred microliters of the cell suspension was added in 15 mL of sodium phosphate buffer at 42 °C containing 1 mg/mL α_{s1}-, α_{s2}-, or β-CN to obtain an $OD_{650\ nm}$ of 1. The samples were incubated at 42 °C, and an aliquot was collected immediately and after 2 and 4 h of incubation. Caffeine was added (3.26 mg/L final concentration) as internal standard for HPLC. Samples were centrifuged (12000*g*, 5 min, 4 °C), filtered through 0.22 μm filters (Millipore, Molsheim, France), and kept at −20 °C.

Peptide Identification by LC-ESI-MS/MS. Nano-LC experiments were performed using an online liquid chromatography tandem mass spectrometry (MS/MS) set up using a nano-LC system (LC-Packings, Dionex, Voisins le Bretonneux, France) fitted to a QSTAR XL (MDS SCIEX, Ontario, Canada) equipped with a nanoelectrospray ion source (ESI) (Proxeon Biosystems A/S, Odense, Denmark). Samples were first concentrated on a PepMap 100 C_{18} column (5 × 0.3 mm, 5 μm particule size, Dionex, Amsterdam, The Netherlands). Peptides were separated on a PepMap C_{18} column (150 × 0.075 mm, 3 μm particule size, Dionex). Solvent A contained 2% (v/v) acetonitrile, 0.08% (v/v) formic acid, and 0.01% (v/v) TFA in deionized water and solvent B, 95% (v/v) acetonitrile, 0.08% (v/v) formic acid, and 0.01% (v/v) TFA in deionized water. A linear gradient from 10 to 50% of solvent B in 65 min was applied for the elution at a flow rate of 0.2 μL/min. Eluted peptides were directly electrosprayed into the mass spectrometer operated in positive mode. A full continuous MS scan was carried out followed by three data-dependent MS/MS scans. Spectra were collected in the range of m/z 450−1500 for MS and in the range of m/z 60−2000 for MS/MS spectra. The three most intense ions from the MS scan were selected individually for collision-induced dissociation (1+ to 4+ charged ions were considered for the MS/MS analysis). The mass spectrometer was operated in data-dependent mode automatically switching between MS and MS/MS acquisition using Analyst QS 1.1 software. The instrument was calibrated by multipoint calibration using fragment ions that resulted from the collision-induced decomposition of the β-CN(f193−209). The peptides present in the samples were identified from MS and MS/MS data by using MASCOT v. 2.2 software against a homemade database dealing with major milk proteins and corresponding to a portion of the Swissprot database. No specific enzyme cleavage was used, and the peptide mass tolerance was set to 0.3 Da for MS and to 0.2 Da for MS/MS. For a database containing 25 protein sequences (N) and a significance level of 0.04 (α), the cutoff score is equal to $-10 \times \log(\alpha/N) = 28$. Matches with a Mascot score superior or equal to this cutoff score were considered to be statistically significant.

RESULTS AND DISCUSSION

The capacity of *S. thermophilus* strains to generate peptides from CNs was evaluated by a biochemical approach (Table 1). The strains were grown to midexponential phase; cell pellets were recovered and further incubated with purified α_{s1}-CN, α_{s2}-CN, or β-CN in a 50 mM sodium phosphate buffer, pH 7.5. After 2 or 4 h of incubation at 42 °C, the peptides contained in the supernatants were separated by HPLC and characterized by

dx.doi.org/10.1021/jf202176d | J. Agric.Food Chem. 2012, 60, 554−565

Table 2. Peptides Generated from Bovine β-Casein by the Proteolytic System of *S. thermophilus* H-Strains[a]

Sequence	Fragment	STRAINS					
		PB302	PB385	ATE 19PB8	4F44	Y4	LMD-9
SLVYPFPGPIPN	57-68						4
VPPFLQPEVM	84-93						4
GVSKVKEAM*	94-102	2 4	2 4	4	4	4	2 4
GVSKVKEAMAPK*	94-105	2 4	2 4	2 4	4	2 4	2 4
VSKVKEAMAPK*	95-105	2 4	2 4	2 4		2	2 4
SKVKEAMAPK*	96-105	2 4	2 4	2		2 4	2
KVKEAMAPK*	97-105	2 4	2 4	2 4	2 4	2 4	2 4
KVKEAMAPKHK*	97-107		2		2		
VKEAMAPK	98-105				4		
VKEAMAPKHK	98-107					4	
EAMAPK*	100-105		4			2	2
EAMAPKHK*	100-107	2 4	2 4	2 4	2 4	2 4	2
EAMAPKHKE	100-108					4	
EAMAPKHKEMPFPKYPVEPFTESQ	100-123				4		
HKEMPFPK*	106-113	2 4	2 4	2 4	2 4	2 4	2 4
HKEMPFPKYPVEPF*	106-119	2 4	2 4	2 4	2 4	2 4	2 4
HKEMPFPKYPVEPFTE	106-121	4					4
HKEMPFPKYPVEPFTESQ*	106-123	2 4	2 4	2 4	4	2 4	2
KEMPFPKYPVEPF*	107-119	4	4	2 4		2 4	2 4
EMPFPKYPVEPF*	108-119	2 4	4	2 4	4	2 4	4
EMPFPKYPVEPFTESQ*	108-123		2		4		2
YPVEPF	114-119						4
TESQSLTL*	120-127		4	4		4	2
SLTLTDVENL*	124-133						2 4
SLTLTDVENLHLPLPL*	124-139			2			
SLTLTDVENLHLPLPLLQ*	124-141	2				2	2
TLTDVENL*	126-133			4		2	2 4
TLTDVENLHLPLPL	126-139						4
TLTDVENLHLPLPLLQ*	126-141	2 4	4	2		2	2 4
TDVENL	128-133						4
TDVENLHLPLPL*	128-139		4	2 4		2	4
TDVENLHLPLPLLQ	128-141	4					
DVENLHLPLPL*	129-139					2	4
NLHLPLPL	132-139						4
LHLPLPLLQ*	133-141	4	4		4	2 4	2 4
HLPLPL*	134-139			4		2	2 4
HLPLPLLQ*	134-141	2 4	4	2 4	4	2	2 4
HLPLPLLQSWM*	134-144	2 4	2 4	2 4	4	2	2 4
LPLPL	135-139						4
LPLPLLQ*	135-141	4		4		2 4	2 4
LPLPLLQSW	135-143						4
LPLPLLQSWM*	135-144			4		2	2 4
LQSWMHQPH	140-148	4					
SWMHQPHQPLPPT*	142-154		4	4			2 4
MHQPHQPLPPT	144-154		4				
HQPHQPLPPT*	145-154	4	4	4		2 4	2 4
VMFPPQSVL*	155-163	4	2 4	2			2 4
MFPPQSVL*	156-163			4		2	
FPPQSVL*	157-163					4	2 4
FPPQSVLSL*	157-165			2 4	4	4	2 4
SVLSLS*	161-166	2 4	2 4	2 4	4	2 4	2 4
SVLSLSQ*	161-167	2 4	2 4	2 4	2 4	2 4	2 4
SVLSLSQS*	161-168			2	4		4
VLSLSQ	162-167				4		
LSQSKVLPVPQ*	165-175	2					
SQSKVLPVPQ*	166-175	2 4	2 4	2 4	2 4	2 4	2 4
SQSKVLPVPQK*	166-176	2 4	2 4	2 4	2 4	2 4	2 4
SQSKVLPVPQKA*	166-177				2 4	4	
SQSKVLPVPQKAVPYPQ *	166-182				2 4		
QSKVLPVPQK*	167-176	2 4	2 4	2 4		2 4	2 4
SKVLPVPQ*	**168-175**	2 4	2 4	2 4	2 4	2 4	2 4
SKVLPVPQK*	**168-176**	2 4	2 4	2 4	2 4	2 4	2 4
SKVLPVPQKA*	168-177		2		2		
SKVLPVPQKAVPYPQ *	168-182				2		
KVLPVPQ*	**169-175**	4	4	2 4	4	2 4	2 4
KVLPVPQK*	169-176			4	4	4	2 4
VLPVPQ*	**170-175**	2 4	2 4	2 4	2 4	2 4	2 4
VLPVPQKAVPYPQ *	170-182	2	2		4		
LPVPQ	171-175			4	4	4	4
LPVPQK	171-176					4	
LPVPQKAVPYPQ	171-182					4	
KAVPYPQ*	**176-182**	2 4	2 4	2 4	2 4	2 4	2 4
KAVPYPQR*	**176-183**	2 4	2 4	2	4	2	2 4
KAVPYPQRDMPIQ*	**176-188**	2 4	2 4	2	2	2	2

dx.doi.org/10.1021/jf202176d | *J. Agric.Food Chem.* 2012, 60, 554–565

Table 2. continued

Sequence	Fragment	STRAINS					
		PB302	PB385	ATE 19PB8	4F44	Y4	LMD-9
KAVPYPQRDMPIQA*	**176-189**	2 4	2 4	2 4	4	2 4	2
KAVPYPQRDMPIQAF	176-190		4				
AVPYPQ*	**177-182**	2 4	2 4	2 4	2 4	2 4	2 4
AVPYPQRDMPIQ*	177-188	2 4	4	2 4		2 4	2 4
AVPYPQRDMPIQA	177-189					4	
VPYPQ*	178-182	4		2 4	4	2 4	2 4
VPYPQRDMPI	178-187					4	4
VPYPQRDMPIQ	178-188					4	
RDMPIQA*	**183-189**	2 4	2 4	2	2 4	2	2 4
RDMPIQAF*	**183-190**	2 4	2 4	2 4	2 4	2 4	2 4
RDMPIQAFL*	**183-191**	2 4	2 4	2 4	2 4	2 4	2 4
RDMPIQAFLL*	**183-192**	4	2 4	2 4	2 4	4	4
RDMPIQAFLLY*	183-193	2	2 4		2 4		
DMPIQAF*	**184-190**	4	2 4	4	2	2 4	2 4
DMPIQAFL*	**184-191**	2	2 4	2 4	2	2 4	2 4
DMPIQAFLL*	**184-192**	4	2 4	2	4	2 4	2 4
MPIQAF	185-190					4	4
MPIQAFL	185-191					4	4
PIQAFLL	186-192					4	4
IQAFLL	187-192					4	4
AFLLYQEPVL*	189-198		2 4				
FLLYQEPVL*	**190-198**	2 4	2 4	2	2	2	2
LLYQEPVL*	**191-198**	2 4	2 4	2 4	2 4	2 4	4
LLYQEPVLGPVR*	**191-202**	2 4	2 4	2	2 4	2	2
LLYQEPVLGPVRGPFPIIV*	**191-209**	2 4	2 4	2	2	2	2
LYQEPVL*	**192-198**	4	2 4	2 4	2 4	2 4	2 4
LYQEPVLGPVR*	**192-202**	2 4	2 4	2	2 4	2	2
LYQEPVLGPVRGPFPIIV*	**192-209**	2 4	2 4	2 4	2 4	2 4	2 4
YQEPV	193-197						4
YQEPVL*	**193-198**	2 4	2 4	2 4	2 4	2 4	2 4
YQEPVLGPV*	**193-201**	2 4	2	2 4	2 4	2 4	2 4
YQEPVLGPVR*	**193-202**	2 4	2 4	2 4	2 4	2 4	2 4
YQEPVLGPVRGPFP*	193-206					2 4	
YQEPVLGPVRGPFPIIV*	**193-209**	2 4	2 4	2 4	2 4	2 4	2 4
QEPVLGPVRGPFPIIV*	**194-209**	2 4	2 4	2	2 4	2 4	2 4
LGPVRGPFPIIV*	198-209		2 4				
GPVRGPFP	199-206	4					4
GPVRGPFPIIV*	**199-209**	2 4	2 4	2 4	2 4	2 4	2 4
VRGPFPIIV*	201-209					2	2
GPFPIIV*	**203-209**	2 4	2 4	4	4	4	2
PFPIIV	204-209			4			
FPIIV	205-209			4			
Number of peptides present after 2 h of incubation		48	52	53	38	61	65
Number of peptides present after 4 h of incubation and absent after 2 h		15	14	15	23	20	23
Number of peptides present after 4 h of incubation		58	61	54	53	61	73
Number of peptides present after 2 h of incubation and absent after 4 h		5	5	14	8	20	15
Total number of peptides formed by the strain (2 h and 4 h)		63	66	68	61	81	88
Number of specific peptides produced (2 h and 4 h)		5	6	4	8	17	20
Peptides average size (residues) after 2 h		10.6	10.3	10.3	10.4	10.1	10.0
± standard deviation (residues)		3.5	3.3	3.4	3.5	3.5	3.4
Peptides average size (residues) after 4 h		10.2	10.3	9.3	10.1	9.5	9.2
± standard deviation (residues)		3.4	3.2	3.1	3.9	3.1	3.0

[a]Peptides were detected after 2 or 4 h of hydrolysis in 50 mM phosphate buffer, pH 7.5. Empty box refers to a lack of detection. The asterisk indicates the peptides that are generated after 2 h of incubation. A peptide is considered to be "specific" when it is produced by a maximum of two different strains and is shaded light gray. The peptides produced by all of the H-strains are shaded dark gray.

MS/MS. The L-strain CNRZ1066, which is *prtS*$^-$, displayed, as expected, no proteolytic activity on the three CNs tested after 2 or 4 h of incubation. As no peptidase activity, from a cell lysis, was detected in the medium in which the cells were incubated, the hydrolysis of caseins observed here resulted only from a surface proteolytic activity.

Identification of Peptides Generated from β-, α_{s2}-, and α_{s1}-Casein Hydrolysis. *Identification of Peptides Generated from β-CN.* Not only was β-CN hydrolyzed more quickly by the proteolytic system of the *S. thermophilus* strains tested, but its hydrolysis degree was also higher. After 2 h of incubation, no hydrolysis was detected with the M-strains (data not shown), whereas 23.1% of the peptide bonds (48 of 208 bonds) of the β-CN were cleaved by all H-strains taken together, resulting in a production of 84 different peptides (Table 2). The mean number of peptides produced by each strain was 52.8 ± 9.6. The number of generated peptides varied from one strain to another because only 38 peptides were liberated by strain 4F44, whereas 65 peptides were generated by strain LMD-9 (Table 2).

After 4 h of incubation, 31.3% of the peptide bonds of the β-CN were hydrolyzed by the 6 H-strains, leading to a total of 109 different peptides. The average number of peptides produced by each H-strain was 60.0 ± 7.2 (Table 2). The proteolytic profiles differed between the strains analyzed. For example, only 53 peptides were detected for strain 4F44, whereas 73 peptides were generated by strain LMD-9 after 4 h of hydrolysis. Strains Y4 and LMD-9 were the most proteolytic

dx.doi.org/10.1021/jf202176d | J. Agric.Food Chem. 2012, 60, 554–565

Table 3. Peptides Generated from Bovine β-Casein by the Proteolytic System of *S. thermophilus* M-Strains[a]

Sequence	Fragment	STRAINS			
		CNRZ 404	CNRZ 445	ATCC 19258	HAD8α
RELEELNVPGEIVE	1 - 14				X
RELEELNVPGEIVESL	1 - 16				X
KIEKFQSEEQQQTEDELQDKIHPF	29 - 52		X		
KIEKFQSEEQQQTEDELQDKIHPFAQTQ	29 - 56		X		
KVKEAMAPK*	97 - 105		X		
EMPFPKYPVEPF*	108 - 119				X
MHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQK	144 - 176	X	X		
SLSQSKVLPVPQK	164 - 176	X	X		
SQSKVLPVPQ*	166 - 175	X		X	X
SQSKVLPVPQK*	166 - 176	X	X	X	
SQSKVLPVPQKAVPYPQRDMPIQA	166 - 189		X		
SQSKVLPVPQKAVPYPQRDMPIQAF	166 - 190		X		
SKVLPVPQK*	168 - 176		X		
AVPYPQRDMPIQA	177 - 189				X
AVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV	177 - 209				X
VPYPQRDMPIQAF	178 - 190	X			
VPYPQRDMPIQAFL	178 - 191	X	X		X
VPYPQRDMPIQAFLLY	178 - 193		X		
RDMPIQAF*	183 - 190			X	X
RDMPIQAFL*	183 - 191				X
DMPIQAFL*	184 - 191				X
FLLYQEPVLGPVRGPFPIIV	190 - 209			X	
LLYQEPVL*	191 - 198				X
LLYQEPVLGPVRGPFPIIV*	191 - 209		X		
LYQEPVL*	192 - 198				X
YQEPVL*	193 - 198			X	X
YQEPVLGPVR*	193 - 202	X	X		
YQEPVLGPVRGPFP	193 - 206	X			X
YQEPVLGPVRGPFPIIV*	193 - 209	X	X	X	X
QEPVLGPVRGPFPIIV*	194 - 209	X	X	X	X
Total number of peptides generated		10	15	7	16
Peptides average size (residues)		15.1	17.9	12.6	12.8
± standard deviation (residues)		6.4	7.2	4.8	6.2
Average size of peptides only generated by M-strains (residues)		18.3	22.1	20.0	19.3
± standard deviation (residues)		8.5	6.7	-	8.0

[a]Peptides were detected only after 4 h of hydrolysis in 50 mM phosphate buffer, pH 7.5. "X" indicates that the peptide is produced by the corresponding strain; empty box refers to a lack of detection. Peptides shaded gray are produced only by M-strains. An asterisk next to the sequence indicates a peptide also generated by all of the H-strains studied. "S" in a sequence indicates a residue of phosphoserine.

toward β-CN because almost all new peptides produced by strains PB302 and PB385 between 2 and 4 h of hydrolysis were already released after 2 h of hydrolysis by strains LMD-9 and Y4. Only 44 of the peptides identified were formed by all of the H-strains studied. Globally, during the hydrolysis of 4 h, 116 different peptides could be produced by the 6 H-strains tested, and only 7 peptides found after 2 h of hydrolysis were not recovered after 4 h (β-CN(f97−107), β-CN(f124−139), β-CN(f124−141), β-CN(f165−175), β-CN(f168−177), β-CN-(f168−182), and β-CN(f201−209); Table 2).

The number of specific peptides (a specific peptide was defined as a peptide produced by a maximum of 2 different strains) varied from one strain to another: strain ATE19PB8 produced only 4 specific peptides, whereas strains Y4 and LMD-9 generated 17 and 20, respectively. Such differences could not be attributed only to the import of peptides which could differ between the strains or to an additional hydrolysis, because few of the peptides generated after 2 h of β-CN hydrolysis disappeared after 4 h (Table 2). Therefore, most of the peptides generated from β-CN after 2 h of hydrolysis by the H-strains corresponded to final peptides. The large proportion of proline residues (1−4) in their sequence could have increased their resistance toward proteolysis.[24]

Only 30 peptides were generated by the M-strains after 4 h of incubation, suggesting that their proteolytic capacities were lower than those of the H-strains (Table 3). Thirteen of the 30 peptides were specifically formed by the 4 M-strains studied in this work (Table 3). The mean size of these peptides was about 21 residues, that is, significantly longer than for H-strains (about 10 residues). Fifteen of 17 peptides generated from β-CN by both M- and H-strains were systematically produced by all of the H-strains studied. Therefore, these peptides were generated from accessible zones of caseins by the proteolytic system of *S. thermophilus* even by the less efficient system of the M-strains. Galia et al.[16] showed that the *prtS* allele of H-strains LMD-9, PB302, and PB385 and of M-strain HAD8α are the same and that M-strains expressed PrtS at lower levels than H-strains in M17 medium. Therefore, a lower proteolytic enzyme expression could slow the kinetics of hydrolysis, and large peptides observed in the case of M-strains might correspond to intermediate peptides.

Cleavage site distribution analysis of the whole β-CN sequence revealed a varied distribution of cleaved bonds (Figure 1). Thus, no peptide bond located between residues 1 and 92 was cleaved after 2 h of incubation. Even after 4 h of hydrolysis, the N-terminal part of the β-CN was very poorly hydrolyzed because only 2 peptide bonds (14−15 and 16−17) were cleaved by M-strain HAD8α, 3 (28−29, 52−53, and 56−57) by M-strain CNRZ445, and 3 (56−57, 68−69, and 83−84) by H-strain LMD-9 (Figure 1). Most of the peptides generated by all of the H-strains came from the 160−209 region of the β-CN. On the basis of hydrophobicity estimated according to the method of Kyle and Doolittle,[25] it appeared in a first approximation that the hydrophilic amino-terminal extremity

dx.doi.org/10.1021/jf202176d | J. Agric.Food Chem. 2012, 60, 554−565

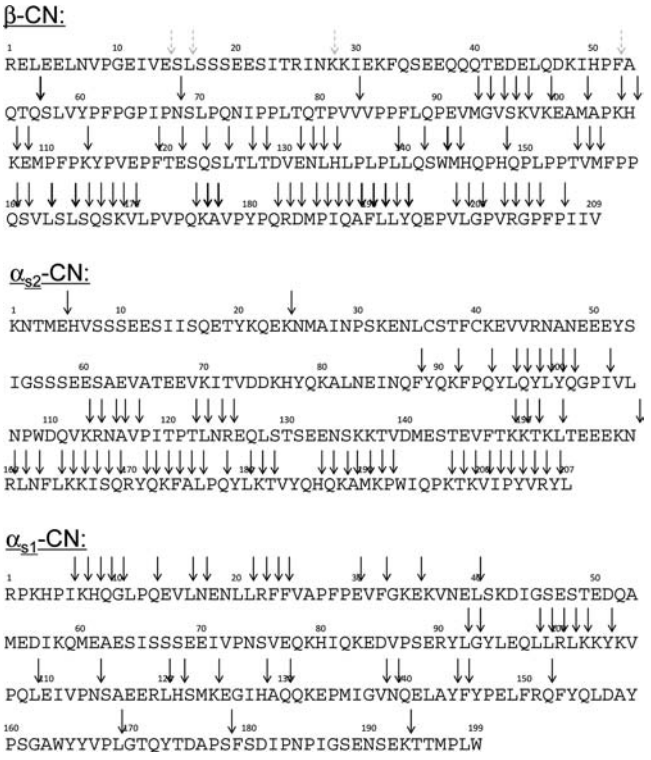

Figure 1. Peptide bonds of bovine β-, α_{s2}-, and α_{s1}-CN hydrolyzed after 4 h of incubation by the proteolytic system of *S. thermophilus* strains. The arrows represent peptide bonds that are hydrolyzed, gray dotted arrows indicate bonds cleaved only by the M-strains tested in this work, and bold arrows indicate bonds cleaved by both M- and H-strains.

of the protein (residues 1−50) was more resistant to hydrolysis than the hydrophobic carboxy-terminal extremity (153−175 and 187−209 residues).

Identification of Peptides Generated from α_{s2}-CN. The H- and M-strains did not generate any hydrolysis products from α_{s2}-CN after 2 h, and only the H-strains produced peptides after 4 h (Table 4). Therefore, α_{s2}-CN appeared to be hydrolyzed at a slower rate than β-CN by *S. thermophilus*. Sixty-two peptide bonds of 206 were hydrolyzed (30.0%) after 4 h of hydrolysis of this CN by the H-strains, leading to the production of 89 different peptides. The mean number of peptides produced by each strain was 35.8 ± 12.4. The mean number of residues was 9.5 ± 3.8. As observed for β-CN, a great variability between the six H-strains tested was observed. Strains PB302 and ATE19PB8 generated only 25 peptides from α_{s2}-CN, whereas strains PB385 and 4F44 produced 45 and 56 peptides, respectively. These two strains were able to produce a great number of specific peptides compared to the other four. Only six of the peptides recovered in the hydrolysis medium were common for the six H-strains studied, and 58.4% of the peptides released appeared to be specific.

As observed for β-CN, the cleavage sites were not uniformly distributed along the α_{s2}-CN sequence (Figure 1). α_{s2}-CN is composed of three regions: a negatively charged region (3−18), a hydrophobic region (78−109), and a positively charged region (126−207). As shown by Tauzin et al.[23] using a tryptic digestion, the association of α_{s2}-CN in a tetrameric form in phosphate buffer (pH 8.1) leads to a protection of some regions toward hydrolysis. Thereby, the carboxy extremity of α_{s2}-CN (153−207) was more susceptible to trypsin hydrolysis. On the contrary, the amino extremity (1−91) and the central region (114−150) were almost not accessible to trypsin. A similar phenomenon was observed with the proteolytic system of *S. thermophilus* H-strains (Figure 1). Indeed, the region 159−207 was extensively hydrolyzed, whereas only the two bonds 5−6 and 24−25 were cleaved in the amino-terminal part of the protein (residues 1−88) by strains 4F44 and LMD-9 (Figure 1; Table 4). Therefore, the most hydrolyzed region of α_{s2}-CN was hydrophilic, and not hydrophobic as previously observed with β-CN. This suggested that the hydrophobicity was not the main parameter to determine CN susceptibility to hydrolysis by the proteolytic system of *S. thermophilus*. Indeed, accessibility to substrate appeared to be a crucial parameter because the CN regions that are not involved in oligomerization were hydrolyzed by the proteolytic system. CN accessibility is also the determinant parameter for the action of the proteolytic system of *Lb. helveticus*.[26] Besides the peptides generated by all H-strains, the specific peptides were also released from accessible zones of caseins, which might appear surprising because the proteolytic system of all bacteria should be able to easily hydrolyze these regions. PrtS is the sole caseinolytic CEP that has been identified in *S. thermophilus*, and the polymorphism concerning this CEP mainly consists of its presence or absence. If PrtS is responsible for the hydrolysis observed, this implies that when the affinity of the CEP for a bond located inside an accessible region is low, the cleavage of this bond requires a higher quantity of the CEP and, therefore, a higher expression of this protease.

Identification of Peptides Generated from α_{s1}-CN. As observed with α_{s2}-CN, the H- and M-strains did not hydrolyze α_{s1}-CN after 2 h, and only H-strains were able to generate peptides after 4 h of hydrolysis (Table 5). α_{s1}-CN was the least hydrolyzed of the three CNs studied because only 39 of the 198 bonds (i.e., 19.7%) were cleaved by the 6 *S. thermophilus* H-strains taken together, leading to the formation of only 40 peptides. The mean number of peptides produced by each strain was 20.0 ± 5.0, and the mean number of residues was 9.6 ± 4.6. Only 20.0% of the peptides were found to be produced by all six H-strains studied, with strain LMD-9 being the most proteolytic strain, as observed on β-CN. This strain generated 29 peptides, whereas strains PB385 and Y4 liberated only 16 peptides. Many of the peptides produced from α_{s1}-CN by the six H-strains were specific, this type of peptide representing 55% of the peptides produced. Half of the specific peptides was produced only by strain LMD-9. Four peptides (α_{s1}-CN(f1−8), α_{s1}-CN(f1−9), α_{s1}-CN(f1−13), and α_{s1}-CN-(f1−17)) have been characterized after milk hydrolysis by a pure culture of *S. thermophilus* CNRZ385.[12] These peptides, except α_{s1}-CN(f1−17), were also generated by all of the H-strains studied in this work and therefore belonged to the group of nonspecific peptides.

Kumosinski et al.[27] described the structure of α_{s1}-CN as follows: a hydrophilic region from residue 1−12, a hydrophobic region from residue 13 to 40 approximately, a hydrophilic region until residue 100, and finally a highly hydrophobic region from residue 100 to the carboxy-terminal part. The α_{s1}-CN monomers could associate by hydrophobic interactions or via the charged regions. Hence, some peptide bonds could become inaccessible to proteolysis in the multimers formed. Analysis of the cleavages observed in our experiments revealed that the regions 90−110 and 1−40 were the most susceptible to hydrolysis as about 40% of the bonds in these regions were cleaved (Figure 1). On the contrary, the region 41−91

Table 4. Peptides Generated from Bovine α_{s2}-Casein by the Proteolytic System of *S. thermophilus* H-Strains[a]

		STRAINS					
Sequence	Fragment	PB302	PB385	ATE19PB8	4F44	Y4	LMD-9
KNTMEHVSSSEESIISQETYKQEK	1-24				X		X
HVSSSEESIISQETYKQEK	6-24				X		
YQKFPQYLQ	89-97	X			X		
YLQYLY	95-100			X	X		
QYLYQGPIVLNPWDQVKRN	97-115					X	
YLYQGPIVLNPWDQVK	98-113				X	X	X
YLYQGPIVLNPWDQVKR	98-114		X	X	X		
YLYQGPIVLNPWDQVKRN	98-115				X		
LYQGPIVLNPWDQVK	99-113		X				
LYQGPIVLNPWDQVKR	99-114		X		X		
LYQGPIVLNPWDQVKRN	99-115				X	X	X
YQGPIVLNPWDQVK	100-113	X	X	X			X
YQGPIVLNPWDQVKR	100-114	X	X	X			X
YQGPIVLNPWDQVKRN	100-115		X			X	
QGPIVLNPWDQVK	**101-113**	X	X	X	X	X	X
QGPIVLNPWDQVKR	**101-114**	X	X	X	X	X	X
QGPIVLNPWDQVKRN	**101-115**	X	X	X	X	X	X
GPIVLNPWDQVKR	102-114			X			X
VLNPWDQVK	105-113						X
VLNPWDQVKR	105-114	X	X	X	X		X
VLNPWDQVKRN	105-115					X	
NAVPITPT	115-122	X				X	
NAVPITPTL	115-123					X	
AVPITPT	116-122			X			
AVPITPTL	**116-123**	X	X	X	X	X	X
AVPITPTLN	116-124				X	X	
AVPITPTLNR	116-125				X	X	X
VPITPTL	117-123			X			X
PITPTLNR	118-125				X	X	X
KTKLTEEEKN	150-159		X				
TKLTEEEKN	151-159	X	X	X			X
KTKLTEEEKNRL	151-161				X		
KTKLTEEEKNRLNFL	151-164				X		
KLTEEEKNRL	152-161				X		
TEEEKNRL	154-161				X		
TEEEKNRLNFL	154-164				X		
RLNFLK	160-165				X		
RLNFLKK	160-166	X	X		X		
LNFLK	161-165			X	X		
NFLKKISQ	162-169				X		
FLKKISQRY	163-171				X		
KISQRYQKF	166-174		X				
ISQRYQKF	167-174		X				
SQRYQKF	168-174				X	X	
QRYQKF	169-174		X				
QKFALPQ	172-178					X	
QKFALPQYLK	172-181					X	X
KFALPQ	173-178				X		X
KFALPQYL	173-180				X		
KFALPQYLK	173-181		X		X		
KFALPQYLKT	173-182				X	X	X
FALPQ	174-178	X	X	X	X		
FALPQYLK	174-181		X				
ALPQYLK	175-181		X		X	X	X
FALPQYLKT	175-182		X		X	X	
LPQYLK	176-181			X	X	X	X
PQYLK	177-181		X		X	X	X
PQYLKT	177-182				X		
YLKTVYQH	179-186	X	X		X		
YLKTVYQHQ	179-187		X				
KTVYQHQKA	181-189		X				
TVYQHQKA	182-189	X	X	X		X	
KAMKPWIQPK	188-197				X	X	X
AMKPWIQPK	189-197	X	X		X		
AMKPWIQPKT	189-198	X	X		X		
AMKPWIQPKTK	189-199	X	X	X	X		
MKPWIQPK	190-197	X	X		X		
MKPWIQPKT	190-198		X		X		
MKPWIQPKTK	190-199	X	X		X		
KPWIQPK	191-197	X	X	X		X	X
KPWIQPKT	**191-198**	X	X	X	X	X	X
KPWIQPKTK	**191-199**	X	X	X	X	X	X
PWIQPK	192-197		X		X		X
PWIQPKT	192-198						X

dx.doi.org/10.1021/jf202176d | J. Agric.Food Chem. 2012, 60, 554–565

Table 4. continued

Sequence	Fragment	STRAINS PB302	PB385	ATE19PB8	4F44	Y4	LMD-9
PWIQPKTK	192-199				X		
WIQPK	193-197		X				
WIQPKTK	193-199	X		X		X	
TKVIP	198-202	X			X	X	
TKVIPY	198-203		X		X	X	
TKVIPYV	198-204		X				
TKVIPYVR	198-205	X	X	X	X	X	
TKVIPYVRY	198-206		X		X		
TKVIPYVRYL	198-207		X	X	X		
KVIPYVRYL	199-207		X		X		
VIPYVR	200-205					X	
VIPYVRYL	200-207					X	
IPYVR	201-205			X			X
IPYVRYL	201-207					X	
PYVRYL	202-207		X				
Total number of peptides generated		25	45	25	56	34	29
Number of specific peptides produced		2	17	6	25	12	8
Peptides average size (residues)		9.4	9.4	9.5	9.8	9.5	10.3
± standard deviation (residues)		2.8	3.2	3.6	4.0	3.6	4.3

[a]Peptides were detected after 4 h of hydrolysis in 50 mM phosphate buffer, pH 7.5. "X" indicates that the peptide is produced by the corresponding strain; empty box refers to a lack of detection. A peptide is considered to be "specific" when it is produced by a maximum of two different strains and is shaded light gray. The peptides produced by all of the strains are shaded dark gray.

Table 5. Peptides Generated from Bovine α_{s1}-Casein by the Proteolytic System of *S. thermophilus* H-Strains[a]

Sequence	Fragment	STRAINS PB302	PB385	ATE19 PB8	4F44	Y4	LMD-9
RPKHPIKH	**1-8**	X	X	X	X	X	X
RPKHPIKHQ	**1-9**	X	X	X	X	X	X
RPKHPIKHQG	1-10		X				
RPKHPIKHQGLPQ	**1-13**	X	X	X	X	X	X
RPKHPIKHQGLPQEVLNENLLRF	1-23		X		X	X	X
KHQGLPQEVL	7-16				X		
HQGLPQ	8-13	X		X	X	X	X
QGLPQEVL	9-16	X		X	X		
QGLPQEVLN	9-17	X	X	X	X		X
GLPQEVLNENLL	10-21		X	X			
EVLNENLLR	14-22	X	X	X		X	X
NENLLR	**17-22**	X	X	X	X	X	X
NENLLRF	**17-23**	X	X	X	X	X	X
NENLLRFF	**17-24**	X	X	X	X	X	X
ENLLR	18-22						X
FFVAPFPEVF	23-32						X
FVAPFPE	24-30						X
FVAPFPEVF	24-32		X	X	X	X	X
FVAPFPEVFGKE	24-35			X			
VAPFPEVF	25-32			X			
GKEKVNEL	33-40			X			X
GYLEQL	**93-98**	X			X		
GYLEQLL	**93-99**	X	X	X	X	X	X
GYLEQLLR	93-100	X			X		
GYLEQLLRL	**93-101**	X	X	X	X	X	X
GYLEQLLRLK	93-102	X	X	X	X	X	
YLEQLL	94-99			X			
YLEQLLRL	94-101			X	X	X	X
YLEQLLRLK	94-102				X	X	
KVPQLEIVPN	105-114						X
EIVPNSAEERLH	110-121						X
SAEERLHSMK	115-124	X		X		X	X
HSMKEGIH	121-128						X
SMKEGIHAQ	122-130	X	X	X	X		X
AQQKEPMIGVN	129-139						X
NQELAY	139-144						X
QELAY	140-144						X
YPELFRQ	146-152						X
GTQYTDAPSFSDIPNPIGSENSEKTTMPLW	170-199						X
FSDIPNPIGSENSEK	179-193						X
Total nb of peptides formed		17	16	22	20	16	29
Nb of specific peptides produced		1	2	5	3	1	13
Peptides average size (residues)		8.4	9.9	8.7	9.1	9.4	9.8
± standard deviation (residues)		1.8	3.9	1.9	3.7	4.0	5.2

[a]Peptides were detected after 4 h of hydrolysis in 50 mM phosphate buffer, pH 7.5. "X" indicates that the peptide is produced by the corresponding strain; empty box refers to a lack of detection. A peptide is considered to be "specific" when it is produced by a maximum of two different strains and is shaded light gray. The peptides produced by all of the strains are shaded dark gray.

dx.doi.org/10.1021/jf202176d | J. Agric.Food Chem. 2012, 60, 554–565

appeared to be very resistant to hydrolysis and the region 152−199 was cleaved only four times by the sole strain LMD-9. As both hydrophobic and hydrophilic regions could be hydrolyzed or, on the contrary, protected from hydrolysis, the accessibility of a region seems to be the most accurate hypothesis to explain the susceptibility to hydrolysis.

Implication of Protease PrtS in the Formation of the Peptides. The CEP of lactococci have been divided into two main groups on the basis of their substrate specificity. The P_I-type proteases hydrolyze predominantly β-CN, and the P_{III}-type proteases also cleave α- and κ-CNs.[28,29] The differences between the two groups are not so clear, and mixed-type specificities have been highlighted. Besides, on the basis of their action on the α_{s1}-CN(f1−23) fragment, Exterkate et al.[30] proposed to divide the proteases of lactococci into seven groups and identified six potential cleavage sites on the α_{s1}-CN(f1−23) fragment. The same cleavage sites have been observed using a purified PrtS from *S. thermophilus* CNRZ385:[13] four were major sites of hydrolysis, one was a medium site, and one was a minor site (Figure 2).

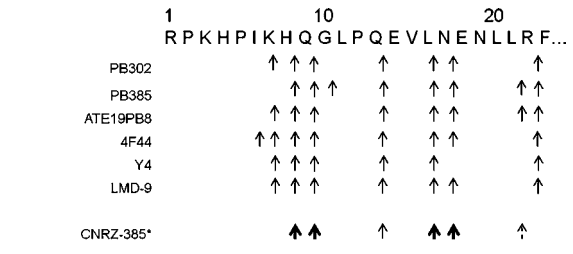

Figure 2. Hydrolysis of the region 1−23 of α_{s1}-CN by the proteolytic system of *S. thermophilus* PB302, PB385, ATE19PB8, 4F44, Y4, and LMD-9 in 4 h in 50 mM phosphate buffer, pH 7.5. The cleavage sites are represented by arrows. CNRZ-385* refers to the specificity of PrtS of the strain CNRZ385, determined on the peptide 1−23 of α_{s1}-CN. Its six major, medium, and minor cleavage sites are indicated by a large, a medium, and a dotted arrow, respectively.[13].

In our experiments, *S. thermophilus* strains were able to degrade β-CN, but also α-CNs at a slower rate, suggesting that their protease(s) displayed intermediate P_I- and P_{III}-types. This observation is consistent with that obtained for the sole CEP of *S. thermophilus* described today, that is, PrtS.[13] The analysis of the cleavage sites in the 1−23 region of the α_{s1}-CN (Figure 2) revealed that they corresponded to those that were observed on the peptide α_{s1}-CN(f1−23) after hydrolysis by PrtS purified from *S. thermophilus* CNRZ385.[13] Four of the six bonds of α_{s1}-CN(f1−23), which have been cleaved by PrtS of the strain CNRZ385, were cut by all of the H-strains studied in this work; they corresponded to three major sites, H^8−Q^9, Q^9−G^{10}, L^{16}−N^{17}, and to the medium site Q^{13}−E^{14} (Figure 2). The major cleavage site N^{17}−E^{18} was cut by all of the H-strains, except strain Y4, and the minor site L^{21}−R^{22} was hydrolyzed by two of the six H-strains. This bond was hydrolyzed at a very slow rate in the study of Fernandez-Espla et al.,[13] and this tendency was also found in our study. The bond R^{22}−F^{23} of the α_{s1}-CN was cut by all six H-strains, whereas PrtS, purified from strain CNRZ385, did not cut this bond in peptide α_{s1}-CN(f1−23). The pure PrtS was unable to generate the cleavage between the residues R22 and F23 in peptide α_{s1}-CN(f1−23), because this bond is the last one and could have been hydrolyzed only if PrtS exhibited an exopeptidase-like activity. Contrarily, this bond being internal in α_{s1}-CN, it was not surprising that it had been cut by an endopeptidase activity.

Three new bonds were also hydrolyzed in our study: I^6−K^7, K^7−H^8, and G^{10}−L^{11}. The cleavage of the I^6−K^7 bond of α_{s1}-CN (hydrolyzed only by strain 4F44) was already reported for *Lb. helveticus* CNRZ32,[31] CNRZ303, and CP790.[28] The K^7−H^8 bond of α_{s1}-CN was hydrolyzed by five of the six H-strains considered. This is the first time that this bond is reported as being cleaved by a CEP of a LAB. Nevertheless, the importance of this cleavage site could not be evaluated because the generated peptides were identified but not quantified. The G^{10}−L^{11} bond was hydrolyzed only by strain PB385.

The correspondence between the major and medium cleavage sites observed with PrtS of strain CNRZ 385 on peptide α_{s1}-CN(f1−23), and those observed with the proteolytic system of the six H-strains studied here on the region 1−23 of α_{S1}-CN (the hydrolysis of the major site N^{17}−E^{18} was lacking only for strain Y4), led to the conclusion that PrtS of these strains was probably responsible for the hydrolysis of CNs reported here. Knowing that strains PB302, PB385, and LMD-9 and M-strain HAD8α possess the same *prtS* allele, how can the fact that strain LMD-9 generated 88 peptides, of which 20 were specific, from β-CN be explained, whereas strains PB302 and PB385 generated 63 and 66 peptides, of which 5 and 6 were specific, respectively? One hypothesis is to consider that all of these strains did not display the same level of expression of PrtS, leading to different enzyme/substrate ratios between the strains. Such an assumption is based on the results of Galia et al.,[16] who observed that the M-strains possessing the *prtS* gene expressed the protease PrtS at a lower level than the H-strains. Thus, if the level of expression of PrtS could vary between the M- and H-strains, it may be possible that it could be also differ within the H-strain group.

***S. thermophilus* Is Able To Release Bioactive Peptides from Caseins.** Hydrolysis of β-, α_{s2}-, and α_{s1}-CN by the proteolytic system of *S. thermophilus* H-strains led to approximately 250 peptides. As the bioactive peptides can exert numerous different effects on the body[3,32] and no rapid method of screening of the different bioactivities exists, the sequence of the peptides obtained in this work was compared to that of the bioactive peptides published in the literature or listed in the "database of biologically active peptide sequences".[33] Among the peptides generated by the tested *S. thermophilus* strains, 13 peptides from β-CN, 5 from α_{s2}-CN, and 2 from α_{s1}-CN were listed as bioactive. Although many bioactive peptides from α_{s1}-CN have been described in the literature, only two bioactive peptides were identified in this study. It must be noted that this work focused on the presence of bioactive peptides and not on their quantification. Thus, some of them can be present in low concentration, but the overall biological activity of a hydrolysate may also result from additive effects.

Angiotensin I-Converting Enzyme (ACE) Inhibitors. Fifteen peptides were ACE inhibitors (Table 6). Nine of these peptides were identified in β-CN hydrolysates, five were identified in α_{s2}-CN hydrolysates, and one was identified in α_{s1}-CN hydrolysates (Table 6). Finally, all of the bioactive peptides resulting from α_{s2}-CN hydrolysis were ACE inhibitors, one being also an antioxidant. It must be noted that the peptides generated from α_{s2}-CN were often specific peptides, generated by one or a maximum of three strains of *S. thermophilus* (Table 4).

Other Bioactive Peptides. Several other potential bioactive peptides were detected in the hydrolysis media of β-CN (Table

Table 6. Potential Bioactive Peptides Generated by the Proteolytic System of Various *S. thermophilus* Strains from Bovine β-, α_{s2}-, and α_{s1}-Caseins after 2 or 4 h of Hydrolysis[a]

source		sequence	strains	activity	ref
β-CN	98−105	VKEAMAPK	4F44	antioxidant	34
	168−175	SKVLPVPQ	all	ACE inhibitor	35
	169−175	KVLPVPQ	all	ACE inhibitor	36
	169−176	KVLPVPQK	ATE19PB8, 4F44,Y4, LMD-9	LOX inhibitor	37
	183−190	RDMPIQAF	all	ACE inhibitor	35
	191−209	LLYQEPVLGPVRGPFPIIV	all	ACE inhibitor and immunomodulating	35, 38
	192−209	LYQEPVLGPVRGPFPIIV	all	mitogene	39
	193−198	YQEPVL	all	ACE inhibitor	40
	193−202	YQEPVLGPVR	all	ACE inhibitor	35, 41
	193−209	YQEPVLGPVRGPFPIIV	all	immunomodulating and antibacterial	2, 42
	199−209	GPVRGPFPIIV	all	ACE inhibitor	43
	201−209	VRGPFPIIV	Y4, LMD-9	ACE inhibitor	44
	203−209	GPFPIIV	all	ACE inhibitor	43
α_{s2}-CN	174−181	FALPQYLK	PB385	ACE inhibitor	45
	189−197	AMKPWIQPK	PB385, PB302, 4F44	ACE inhibitor	36
	190−197	MKPWIQPK	PB385, PB302, 4F44	ACE inhibitor	36
	198−202	TKVIP	PB302, Y4, 4F44	ACE inhibitor	36
	202−207	PYVRYL	PB385	ACE inhibitor and antioxidant	46
α_{s1}-CN	1−9	RPKHPIKHQ	all	ACE inhibitor	47
	1−23	RPKHPIKHQGLPQEVLNENLLRF	LMD-9, PB385, 4F44, Y4	immunomodulating and antibacterial	48

[a]"all" means that the peptide was produced by the six H-strains tested in this study. ACE, angiotensin-converting enzyme (ACE transforms angiotensin I to angiotensin II and also hydrolyzes bradykinin into inactive peptides); LOX, lipoxygenase (lipoxygenase is a dioxygenase that catalyzes oxygenation of certain polyunsaturated fatty acids).

6). Thus, the peptide β-CN(f98−105) displays an antioxidant activity[34] and the peptide β-CN(f169−176) inhibits the lipoxygenase activity.[37] Some of the highlighted bioactive peptides may act on the immune system because the peptide β-CN(f193−209) was described as both an immunomodulator and an antibacterial[2,42] and the peptide β-CN(f191−209), already described as an ACE inhibitor, was also characterized as an immunomodulating peptide.[38] The peptide α_{s1}-CN(f1−23), which has been first described by Lahov and Regelson[48] as a antimicrobial peptide called "isracidin", is also considered to be an immunomodulating peptide.[2] Finally, the peptide β-CN(f192−209) was labeled as potentially mitogenic.[39]

The potential bioactive peptides cited above were generated by all of the H-strains tested, except isracidin, β-CN(f98−105), and β-CN(f169−176). Isracidin was produced by strains PB385, 4F44, Y4, and LMD-9, β-CN(f98−105) by strain 4F44, and β-CN(f169−176) by strains ATE19PB8, 4F44, Y4, and LMD-9 (Table 6). The peptide β-CN(f191−209) present at 2 h of hydrolysis was further degraded by the four strains ATE19PB8, 4F44, Y4, and LMD-9.

Thus, all bioactive peptides, except one, highlighted in this work appeared to be resistant to hydrolysis by the proteolytic system of *S. thermophilus*, confirming the results of Somkuti and Paul,[49] who showed that isracidin remained intact after 4 h of incubation with *S. thermophilus* strains but was degraded by exposure to *Lb. delbrueckii* ssp. *bulgaricus* strains.

Numerous studies have been conducted on bioactive peptides generated from fermentations with *Lactococcus* or *Lactobacillus* strains. Studies on the release of bioactive peptides (or their precursors) from CNs by *S. thermophilus* in coculture with *Lactobacillus* (with the possible addition of enzymes) were also carried out.[7,50,51] To our knowledge, very few studies dealt with the genesis of peptides from the hydrolysis of CNs with *S. thermophilus* alone.[12] Among the bioactive peptides released by *S. thermophilus*, some of them are also generated from CNs by other LAB. Thus, the peptides β-CN(f191−209), β-CN(f192−209), and β-CN(f199−209) are produced by *Lc. lactis*, the peptide α_{S1}-CN(f1−9) is produced by *Lb. helveticus*, and the peptides β-CN(f168−175), β-CN(f169−175), and β-CN-(f183−190) are produced by both bacteria.[52−55] We considered only the bioactive peptides with a proven activity, especially those listed in the "database of biologically active peptide sequences",[33] but the real number of bioactive peptides may be higher. Moreover, the peptides with m/z < 400 were not identified by the LC-ESI-MS/MS analysis, which explains that the reported peptides have a sequence of at least five residues. The identified peptides could also be the precursors of bioactive peptides that could be released in vivo by gastrointestinal enzymes. Thus, Parrot et al.[56] suggested that digestive enzymes diminished the ACE inhibition activity of the peptides detected in Emmental cheese, whereas the digestion of peptides of higher molecular weight would generate new ACE inhibitory peptides. New techniques in vitro would be required to rapidly evaluate the biological activity of a hydrolysate, but finally, only tests on animals would give a global knowledge of the bioactive effect taking into account the presence of antagonist(s) or agonist(s).

The aim of this work was to determine (i) whether the proteolytic system of *S. thermophilus* is able to generate bioactive peptides from some bovine CNs; (ii) whether such a capacity could vary among the *S. thermophilus* strains; and (iii) whether the CEP PrtS could account for such a production, at least in part. The results obtained showed that *S. thermophilus* is able to generate numerous peptides from CNs and that some of them are strain-specific. Among the peptides, several have bioactive potential, leading to the conclusion that *S.*

thermophilus, which is widely used in dairy product manufacture, could be used for the release of bioactive peptides from CNs during processing. Finally, we showed that the protease PrtS could account at least in part for the observed production of the peptides from CNs and, so, for the bioactive peptides detected.

This work poses several questions. The first concerns the origin of the specific peptides that are produced by few strains. Is PrtS responsible for this variability and how? The second question concerns the stability of the bioactive peptides during the storage of the products and their bioavailability for the consumer. Finally, the third concerns the real number of bioactive peptides that are generated by the *S. thermophilus* strains and the possibility of driving their liberation by modifying the proteolytic system of *S. thermophilus*.

Abbreviations Used

ACE, angiotensin I-converting enzyme; CN, casein; H, high acidifying; L, low acidifying; LAB, lactic acid bacteria; M, medium acidifying.

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Funding

This work was supported by Mission scientifique Syndifrais – CNIEL.

ACKNOWLEDGMENTS

We thank Oun-Ki Chang for his valuable assistance and Maira Junjua for her critical reading and corrections of our manuscript.

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