Synergistic Effect Between Different Milk-Derived Peptides and Proteins

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ABSTRACT

Antimicrobial peptides derived from food proteins constitute a new field in the combined use of antimicrobial agents in food. The best examples of milk-derived peptides are those constituted by bovine lactoferricin [lactoferrin f(17–41)] (LFcin-B) and bovine α_{s2} -casein f(183–207). The aim of this work was to study if the antimicrobial activity of a natural compound employed in food preservation, nisin, could be enhanced by combination with the aforementioned milk-derived peptides. Furthermore, the possibility of a synergistic effect between these peptides and bovine lactoferrin (LF) against *Escherichia coli* and *Staphylococcus epidermidis* was also studied. Finally, the most active combinations were assayed against the foodborne pathogens *Listeria monocytogenes* and *Salmonella choleraesuis*. Results showed a synergistic effect when LFcin-B was combined with bovine LF against *E. coli*. In the same way, the combination of LFcin-B with bovine LF was synergistic against *Staph. epidermidis*. Bovine LF and nisin increased their antimicrobial activity when they were assayed together with bovine α_{s2} -casein f(183–207). It is important to note the synergistic effect among LFcin-B and bovine LF, because both compounds might be simultaneously in the suckling gastrointestinal tract and could, therefore, have a protective effect on it. The other synergistic effect highlighted is that between α_{s2} -casein f(183–207) and nisin against *L. monocytogenes* because of the ability of *L. monocytogenes* to develop resistance to nisin.

Key words: synergism, milk-derived antibacterial peptide, antibacterial milk protein

INTRODUCTION

Food preservation procedures such as pasteurization, refrigeration, canning, modified atmosphere packaging, or the incorporation of chemical preservatives in food are usually employed to prevent the growth of bacteria that may cause human disease or food spoilage. Chemi-

cal preservatives such as benzoates, sorbates, nitrites, and sulfites have been used effectively, but their safety is continually under study (Knekt et al., 1999; McCann et al., 2007). The consumer demand for minimally processed foods has led to the search for biopreservatives that can be safely incorporated into various food products. Although numerous studies have shown the effectiveness of biopreservatives against microorganisms (Altieri et al., 2005; Schnurer and Magnusson, 2005), some of them have a limited spectrum of activity, high application cost, or negative effect on the organoleptic quality of foods (Dufour et al., 2003). These limitations can, to an extent, be overcome by combinations of different antimicrobial agents (Zapico et al., 1998; Brannen and Davidson, 2004), combinations of antimicrobials with chelating agents (Stevens et al., 1991), or by the use of antimicrobials together with preservative treatments such as high hydrostatic pressure, low pH, or freeze-thaw cycles (Roberts and Hoover, 1996; García-Graells et al., 2000; Cressy et al., 2003).

Nisin is a bacteriocin produced by *Lactococcus lactis* spp. *lactis* that is primarily active against gram-positive bacteria, and it has found practical application as a food preservative in several food products (Delves-Broughton et al., 1996). The practical application of nisin, however, is limited because of its low stability, reduced activity at high pH, and poor efficacy in certain food matrices (Pol and Smid, 2000).

Lactoferrin (LF) is a key element of the innate host defense system, and, as such, it has crucial antimicrobial activities against a broad range of pathogens. In the case of bacteria, LF affects many gram-positive and gram-negative pathogens (Valenti and Antonini, 2005). In contrast, it seems to promote the growth of beneficial bacteria such as *Lactobacillus* and bifidobacteria (Sherman et al., 2004). The large-scale preparation of LF from cheese whey or skim milk makes it available for human and animal health purposes and commercial applications. Lactoferrin is also used in food preservation by limiting the growth of microbes. For example, incorporation of bovine LF into edible films has a great potential to enhance the safety of foods, or it can also be directly used as a spray applied to beef carcasses (Taylor et al., 2004).

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Antimicrobial peptides derived from food proteins constitute a new field in the use of antimicrobial agents in food. Some of them have shown potent antimicrobial activity and a broad spectrum against gram-positive and gram-negative microorganisms. Antimicrobial peptides have been isolated from various food proteins, but the greatest number described to date are from milk (López-Expósito and Recio, 2006) or from chicken egg white (Ibrahim et al., 2000; Pellegrini et al., 2004). One of the most potent milk-derived antimicrobial peptides described so far corresponds to a fragment of the whey protein LF, named lactoferricin (Bellamy et al., 1992), which possesses an antimicrobial potency against a wide range of microorganisms, which is 10-fold greater than that of the parent protein. Another peptide with a strong antimicrobial activity against gram-positive and gram-negative microorganisms is that corresponding to the bovine α_{s2} -casein f(183–207). This fragment was obtained by hydrolysis of the bovine α_{s2} -casein with pepsin (Recio and Visser, 1999b). Although only few works deal with the synergistic effect of LF with other antimicrobial compounds such as monolaurin, lysozyme, or EDTA (El-lison and Giehl, 1991; Branen and Davidson, 2004), to our knowledge, no synergism has been described among milk-derived peptides and nisin and LF.

The aim of this work was to study whether the peptides α_{s2} -casein f(183–207) and bovine lactoferricin (**LFcin-B**) can exert a synergistic effect in combination with other food proteins and peptides toward selected foodborne pathogens and spoilage bacteria. We intended to evaluate if these 2 antibacterial peptides were able to destabilize the outer membrane of gram-negative microorganisms, to facilitate access of antimicrobial agents with a limited spectrum of activity against gram-negative microorganisms such as LF and nisin.

MATERIALS AND METHODS

Bacterial Strains and Growth Media

Escherichia coli ATCC 25922 was from the American Type Culture Collection (Rockville, MD), and *Listeria monocytogenes* CECT 934, *Staphylococcus epidermidis* CECT 231, and *Salmonella choleraesuis* ssp. *choleraesuis* CECT 4594 were from the Spanish Type Culture Collection (Colección Española de Cultivos Tipo, Valencia, Spain). Tryptic soy broth (**TSB**), tryptic soy agar (**TSA**), brain-heart infusion agar (**BHIA**), and brain-heart infusion (**BHI**) were from Scharlau (Barcelona, Spain). Unless otherwise stated, all other chemicals were of the highest grade commercially available.

Chemicals

Bovine LF was kindly donated by Domo Food Ingredients (Beilen, the Netherlands). Iron content of the LF

preparation was determined by inductively coupled plasma-optical emission spectrometry (Larrea et al., 1997). Nisin (2.5% nisin) was purchased from Sigma (St. Louis, MO).

α_{s2} -Casein f(183–207) and LFcin-B Preparation

Bovine α_{s2} -casein f(183–207) was prepared by conventional fluorenyl-methoxy-carbonyl solid-phase synthesis method with a 431A peptide synthesizer (Applied Biosystems Inc., Überlingen, Germany) and purified after synthesis by semipreparative reversed-phase HPLC (**RP-HPLC**) with the conditions described previously by López-Expósito et al. (2006a).

The LFcin-B was prepared as described previously by Recio and Visser (1999a). Briefly, an LF hydrolysate (5% wt/vol) was prepared in acidified water (pH 3.0) with 3% (wt/wt) of porcine pepsin A (EC 3.4.23.1, 445 U/mg of solid, Sigma) for 4 h at 37°C. The reaction was terminated by heating at 80°C for 15 min, and the pH was adjusted to 7.0 by the addition of 1 M NaOH. The supernatant obtained after centrifugation ($16,000 \times g$ for 15 min) was injected onto a column (150×26 mm i.d.) of SP-Sepharose Fast Flow resin (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated at 4°C with ammonium hydrogen carbonate buffer acidified with formic acid to pH 7.0. Peptides were eluted with a flow rate of 5 mL/min with a gradient going from 0 to 100% in 70 min of 5 M ammonia solution. Finally, the column was eluted with 2 M NaCl, and this fraction containing LFcin-B was desalted by a semipreparative RP-HPLC step. The purity of the LFcin-B obtained was evaluated by RP-HPLC-MS as described previously (López-Expósito et al., 2006b).

Antimicrobial Activity

Antimicrobial activity was determined using Cryo-Tubes Vials (Nunc, Roskilde, Denmark). Single colonies of bacteria grown on TSA plates (*E. coli*, *S. choleraesuis*, and *Staph. epidermidis*) or BHIA plates (*L. monocytogenes*) were inoculated with 10 mL of TSB or BHI and grown overnight at 37°C. A total of 300 μ L of bacterial suspension was diluted 1/50 with TSB or BHI. Bacteria were grown at 37°C, and logarithmic phase organisms were harvested at a density of 1 to 4×10^8 cfu/mL. The culture was then centrifuged at $2,000 \times g$ for 10 min. Bacteria were washed twice with 10 mM Na-phosphate buffer, pH 7.4, and adjusted to 10^5 cfu/mL approximately. A total of 50 μ L of the bacterial suspension was mixed with 50 μ L of the antimicrobial sample to be investigated together with 100 μ L of 2% TSB or BHI in 10 mM phosphate buffer, pH 7.4, and with 800 μ L of 10 mM phosphate buffer, pH 7.4. The mixture was incu-

Table 1. Antibacterial activity expressed as the concentration (μM) of antimicrobial agent that gave a log (N_0/N_f) value between 0.25 and 0.5 against different gram-negative (*Escherichia coli*, *Salmonella choleraesuis*) and gram-positive (*Staphylococcus epidermidis*, *Listeria monocytogenes*) microorganisms for each antimicrobial agent evaluated¹

Antimicrobial	Gram-negative		Gram-positive	
	<i>E. coli</i>	<i>S. choleraesuis</i>	<i>Staph. epidermidis</i>	<i>L. monocytogenes</i>
LF	0.075	1.25	2.500	0.25
Nisin	0.500	5.00	0.050	0.25
f(183–207)	1.250	0.5	2.500	0.05
LFcin-B	0.0125	ND ²	0.050	ND

¹Molecular masses considered were 77,000 for lactoferrin (LF), 3,475 for nisin, 3,115 for α_{s2} -casein f(183–207), and 3,125 for bovine lactoferricin (LFcin-B).

²ND = not determined.

bated at 37°C for 2 h with agitation and then plated on TSA or BHIA plates. The plates were incubated at 37°C (*E. coli*, *S. choleraesuis*, *Staph. epidermidis*) or 30°C (*L. monocytogenes*) for 24 h before the colonies were counted. The assays were conducted in triplicate. The antimicrobial activity was expressed as the concentration of antimicrobial agent that gave a log (N_0/N_f) value between 0.25 and 0.5.

Evaluation of Synergy

To determine antimicrobial interactions, a synergy index was defined based on fractional inhibitory concentration-index (FIC index) described previously by Davidson and Parish (1989). The synergy index of an individual antimicrobial compound is the ratio of the concentration of the antimicrobial compound in an inhibitory combination with a second compound to the concentration of the antimicrobial by itself as follows:

$$\text{Index}_A = (\text{activity of A with B})/\text{activity of A}.$$

The synergy index was calculated as follows with the indices for the individual antimicrobials: synergy index = $\text{Index}_A + \text{Index}_B$. If the synergy index is <1, the interaction is considered to be synergistic; if the synergy index = 1, the interaction is additive; and a synergy index >1 represents antagonism between 2 substances.

RESULTS AND DISCUSSION

Determination of the Antibacterial Activity

The antibacterial activity of the protein and peptides under investigation was determined against 2 gram-negative and 2 gram-positive bacterial strains, and the results are shown in Table 1. The lantibiotic nisin was active against both gram-negative and gram-positive bacteria, although against the gram-negative microorganisms, it showed notably lower activity than against

the gram-positive ones. Nisin is an antimicrobial compound with a spectrum limited essentially to gram-positive microorganisms. Our results showed that nisin could also exert certain antimicrobial activity against gram-negative bacteria, confirming the results reported by Kuwano et al. (2005). The disparities found by different authors are probably due to the conditions of the antibacterial assay. In any case, the results reported in Table 1 show that to reveal some appreciable antibacterial activity against gram-negative bacteria, nisin must be assayed at least at a concentration 10 times higher than against gram-positive bacteria.

The LF was active against both gram-negative and gram-positive bacteria. The LF preparation used in this study contained $198 \pm 5 \mu\text{g}$ of iron/g of dry weight as determined by elemental analysis (i.e., approximately 13.6% saturation, considering 2 metal binding sites per 77,000 Da). The antibacterial activity values ranged from 0.075 μM against *E. coli* to 2.5 μM against *Staph. epidermidis*. In fact, regarding gram-negative bacteria, LF was strongly active against *E. coli* and to a lesser extent against *S. choleraesuis*. Among gram-positive bacteria, *L. monocytogenes* was highly sensitive to LF, whereas *Staph. epidermidis* was only weakly affected by the action of this protein. Thus, the differences observed cannot be easily explained in terms of a different composition of the bacterial membrane. These results are in agreement with previous studies with human LF in which nonenteropathogenic strains of *E. coli* were classified as LF-sensitive strains and *Staph. epidermidis* was relatively resistant to the effect of apo-LF (Arnold et al., 1980). The peptide fragment of LF, LFcin-B, was active against both gram-negative and gram-positive bacteria. Its antibacterial activity was stronger than that of its parent protein, confirming the results reported by other authors (Bellamy et al., 1992). As shown in Table 1, similar activity was previously found for LFcin-B against *E. coli* and *Staph. epidermidis* (Jones et al., 1994).

The peptide f(183–207) derived from α_{s2} -casein was active against both gram-negative and gram-positive

Table 2. Antibacterial activity expressed as the concentration (μM) of antimicrobial agent that gave a log (N_0/N_t) value between 0.25 and 0.5 and synergy indexes (index) for each combination assayed against *Escherichia coli* ATCC 25922 and *Staphylococcus epidermidis*¹

Antimicrobial	<i>E. coli</i>			<i>Staph. epidermidis</i>		
	Activity	Index	Effect	Activity	Index	Effect
LFcin-B with						
LF	0.0075	0.68	Synergism	0.0025	0.51	Synergism
Nisin	0.0500	4.02	Antagonism	0.0250	1.00	Additive
α_{s2} -Casein f(183–207) with						
LF	0.025	0.35	Synergism	0.0250	0.02	Synergism
Nisin	2.500	7.00	Antagonism	0.0050	0.10	Synergism
α_{s2} -Casein f(183–207) with	<i>Salmonella choleraesuis</i>			<i>Listeria monocytogenes</i>		
LF	2.5	1.75	Antagonism	0.0025	0.60	Synergism
Nisin	0.0625	5.50	Antagonism	0.0025	0.60	Synergism

¹Combinations of α_{s2} -casein f(183–207) with lactoferrin (LF) and nisin were also assayed against *Salmonella choleraesuis* and *Listeria monocytogenes*. LFcin-B = bovine lactoferricin.

bacteria. However, similarly to LF, notable differences were observed in the bactericidal activity against the strains investigated. *Listeria monocytogenes* was the strain most sensitive to the action of f(183–207), whereas *Staph. epidermidis* was the least.

Interactions Between LFcin-B and Other Antimicrobial Compounds

To investigate a possible synergistic effect between LFcin-B and LF or nisin against *E. coli* and *Staph. epidermidis*, synergy indices were calculated. Results are shown in Table 2. From the results obtained, it must be highlighted that LF and the LF-derived peptide, LFcin-B, acted synergistically against *E. coli* and *Staph. epidermidis*. The LFcin-B and LF displayed against *E. coli* activity values of 0.0125 and 0.075 μM , respectively, whereas the activity value decreased to 0.0075 μM when they were assayed together. The synergy index determined for the combination LF and LFcin-B against *E. coli* and *Staph. epidermidis* was 0.68 and 0.51, respectively (Table 2). The LF and LFcin-B used in this study were of bovine milk, but if this synergism could also be demonstrated with LFcin and LF from human origin, it could have physiological implications. It has been demonstrated by mass spectrometry that significant amounts of fragments that contain LFcin-B are produced in human stomach following ingestion of LF, and therefore, functional quantities of human LFcin might be generated in the human stomach (Kuwata et al., 1998a). Additionally, LFcin has also been detected in the gastrointestinal tract of adult mice (Kuwata et al., 1998b). In the same way, it was demonstrated that a portion of ingested LF is incompletely hydrolyzed (Spik et al., 1982), and the concentration of LF in human milk is approximately 2 g/L in mature milk (Lönnerdal, 2003)

and 7 g/L in human colostrum (Ward and Conneely, 2004). It is, therefore, likely that LF and LFcin coexist in the gastrointestinal tract of the breast-fed infants, and these compounds could act synergistically, increasing the defenses of the host against invading microorganisms.

When LFcin-B was combined with nisin, an antagonistic effect was found against *E. coli* (FIC index of 4.02), whereas the synergy index achieved against *Staph. epidermidis* revealed an additive interaction (synergy index of 1.0). This antagonistic effect was also previously reported when nisin was combined with reuterin against gram-negative microorganisms (Arqués et al., 2004a).

Interactions Between Bovine α_{s2} -Casein f(183–207) and Other Antimicrobial Compounds

As shown in Table 2, the synergy indices obtained with the α_{s2} -casein peptide combined with LF and nisin revealed a synergistic effect against *Staph. Epidermidis*. Particularly efficient were the combinations of the α_{s2} -casein peptide with LF and nisin against *Staph. epidermidis*, with synergy values of 0.02 and 0.1, respectively. These low indices indicate a strong synergism of these 2 combinations. As can be observed from Figure 1, when both substances were tested alone, concentrations of 10 μM of LF and 5 μM of the α_{s2} -casein peptide were required to reach the maximum growth inhibition. When the combination of LF and the α_{s2} -casein peptide was assayed, a concentration of 2.5 μM of each compound was enough to obtain the same effect. If the α_{s2} -casein peptide could be generated upon enzymatic hydrolysis in the suckling gastrointestinal tract, this synergism might also have a physiological meaning, because both compounds could coexist in the gastrointestinal tract of a breast-fed infant. On the other hand, the synergy

Figure 1. Antibacterial activity at different concentrations of lactoferrin (■), α_{s2} -casein f(183–207) (◆), and lactoferrin + α_{s2} -casein f(183–207) (▲) against *Staphylococcus epidermidis* CECT 231 growth in tryptic soy broth. Antibacterial activity was calculated as $\log N_0/N_f$, where N_0 refers to the control number of colonies without antibacterial material (10^3 cfu/mL) and N_f refers to the number of colonies containing antibacterial compounds after an incubation period of 2 h at 37°C.

between α_{s2} -casein f(183–207) and nisin could find some application in the food industry, in which nisin is already used as a food preservative. Other authors have obtained a synergistic interaction by combining nisin with monolaurin (Mansour and Milliére, 2001), garlic extract (Singh et al., 2001), lactoperoxidase system (Zapico, et al., 1998), or reuterin (Arqués et al., 2004b), but to date, the interaction of nisin with other milk proteins and peptides has not been attempted. Against *E. coli*, only the combination of the casein-derived peptide with LF demonstrated a synergistic interaction, whereas combination with nisin had an antagonistic effect. It had been previously reported that LF in combination with monolaurin inhibited growth of *E. coli* O157:H7 but not *E. coli* O104:H21 (Branen and Davidson, 2004), and therefore, this synergistic behavior should be confirmed with other *E. coli* strains.

Bovine α_{s2} -Casein f(183–207) Interactions Against Foodborne Pathogens

Combinations with synergy indices lower than 0.5 were also assayed against the foodborne pathogens *S. choleraesuis* and *L. monocytogenes*. The results obtained for the combinations of α_{s2} -casein f(183–207) with LF and nisin are shown in Table 2. These 2 combinations were synergistic against *L. monocytogenes* but had an antagonistic effect when tested against *E. coli*. Of special interest was the combination of the peptide from α_{s2} -casein with nisin because of the ability of *L. monocytogenes* to develop resistance to nisin (Davies and Adams,

1994). Probably, the peptide α_{s2} -casein f(183–207) could destabilize the bacterial membrane, making this microorganism more susceptible to the action of nisin. Therefore, as indicated above, the combination of α_{s2} -casein f(183–207) and nisin could be of use in the food industry as a food preservative.

In relation to *S. choleraesuis*, none of the combinations assayed were synergistic against this bacterium. The synergy index was 1.75 for the combination with LF and 5.50 for the combination with nisin (Table 2). The reason why these 2 combinations, casein-derived peptide with LF or nisin, were synergistic against the gram-positive bacteria (*Staph. epidermidis* and *L. monocytogenes*) but not against gram-negative bacteria (*E. coli* and *S. choleraesuis*) is not clear. It may be due to the more complex membrane structure of gram-negative bacteria. However, combinations of LF with Lfcin-B or with the casein-derived peptide exerted a synergistic effect against *E. coli*. It has been postulated that differences in the antibacterial action of EDTA-nisin combinations against different gram-negative bacteria could be attributed to differences in the outer membrane or LPS structure, which may affect the amount of LPS released from the outer membrane and the resulting increase in permeability (Branen and Davidson, 2004).

CONCLUSIONS

The antimicrobial activity of LF and nisin can be enhanced by simultaneous addition of the peptides Lfcin-B and α_{s2} -casein f(183–207). More specifically, these 2 peptides have been demonstrated to act synergistically or additively with LF and nisin against the gram-positive microorganism *Staph. epidermidis*. However, against gram-negative *E. coli*, only the combination of these 2 peptides with LF have proved to be more effective at inhibiting bacterial growth than either agent used alone. Peptide α_{s2} -casein f(183–207) synergistically enhanced the activity of nisin and LF against *L. monocytogenes*. Some of these combinations, such as LF with Lfcin-B or LF with α_{s2} -casein f(183–207), may be relevant for the host defense properties of LF. The results obtained in this work further highlight the potential of using nisin in combination with α_{s2} -casein f(183–207) to improve its effectiveness at inhibiting *L. monocytogenes*. Although results obtained in growth media cannot be directly extrapolated to food matrices, this combination may, therefore, be promising for use in food preservation.

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