Altered Specificity of Lactococcal Proteinase P_I (Lactocepin I) in Humectant Systems Reflecting the Water Activity and Salt Content of Cheddar Cheese

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By using various humectant systems, the specificity of hydrolysis of α_{s1} -, β -, and κ -caseins by the cell envelope-associated proteinase (lactocepin; EC 3.4.21.96) with type P_I specificity (i.e., lactocepin I) from *Lactococcus lactis* subsp. *lactis* BN1 was investigated at water activities (a_w) and salt concentrations reflecting those in cheddar type cheese. In the presence of polyethylene glycol 20000 (PEG 20000)-NaCl ($a_w = 0.95$), hydrolysis of β -casein resulted in production of the peptides comprising residues 1 to 6 and 47 to 52, which are characteristic of type P_{III} enzyme activity (lactocepin III) in buffer. The fragment comprising residues 1 through 166, inclusive (fragment 1-166), which is typical of lactocepin I activity in buffer systems, was not produced. Similarly, peptide 152-160 from κ -casein, which is usually produced in aqueous buffers exclusively by lactocepin III, was a major product of lactocepin I. Most of the specificity differences obtained in the presence of PEG 20000-NaCl were also obtained in the presence of PEG 20000 alone ($a_w = 0.99$). In addition, α_{s1} -casein, which normally is resistant to lactocepin I activity, was rapidly hydrolyzed in the presence of PEG 20000 alone. Hydrolysis of casein in the presence of PEG 300-NaCl or glycerol-NaCl (both having an a_w of 0.95) was generally as expected for lactocepin I activity except that β -casein peptide 47-52 and κ -casein fragment 1-160 were produced; both of these are normally formed by lactocepin III in buffer. The differences in lactocepin specificity obtained in the humectant systems can be attributed to a combination of a_w and humectant hydrophobicity, both of which are parameters that are potentially relevant to the cheese-ripening environment.

Hydrolysis of casein by the cell envelope-associated proteinase of lactococcal starter bacteria, referred to below as lactocepin (EC 3.4.21.96), is the first step in the provision of amino acids essential for starter growth in cheese milk (19). (The trivial name lactocepin, derived from lactococcal cell envelope-associated proteinase (20), is now the International Union of Biochemistry and Molecular Biology (IUBMB)-recommended name; the different specificity types are identified as types I, III, and I/III.) Peptides produced by lactocepin are further processed by an array of intracellular peptidases, which yields small peptides and amino acids which can be utilized by the cell. These products can also impart flavor characteristics to cheese either directly or indirectly as flavor compound precursors (24). It is estimated that starter proteolysis accounts for 80% of the precursors of protein-related flavor compounds (2).

Early studies on casein hydrolysis in aqueous buffers resulted in the classification of lactocepins into two specificity groups (25), type I and type III. Lactocepin I hydrolyzes β -casein and (to a lesser extent) κ -casein, but not α_{s1} -casein. In contrast, lactocepin III readily hydrolyzes both β - and κ -caseins with a peptide bond specificity different from that of lactocepin I and also catalyzes hydrolysis of α_{s1} -casein. The most recent refinement in the classification of lactocepin types is based both on specificity toward the 23-residue N-terminal fragment of α_{s1} -casein and on the identities of nine amino acid residues identified in the enzyme's primary structure which are thought to be involved in substrate binding (4). On the basis of

these criteria, different lactocepin types were classified into seven groups designated groups a to g.

In order to define the pool of peptides with potential to affect the flavor of cheese, a detailed knowledge of the products resulting from the action of lactocepin on casein is required. A considerable amount of information is now available on the specificity of action of lactocepin on various caseins in simple aqueous buffers (12, 15–17, 21–23, 26–28). While the effects of two important cheese parameters, pH and salt concentration, on the action of lactocepin on chymosin-generated α_{s1} - and β -casein fragments have been investigated (3, 5), the cheese environment is, in fact, a complex environment consisting of protein, milkfat, minerals, and water, in which a low water activity (a_w) prevails (8). Although a_w is known to influence enzyme action significantly (7, 9, 10, 18), its effect on the action of lactocepin has not been studied previously.

In the present study, three nonelectrolyte a_w depressors (humectants) were used in combination with NaCl to produce systems in which the a_w and salt concentration were equivalent to the a_w and salt concentration measured in cheddar cheese. The peptides resulting from hydrolysis of α_{s1} -, β -, and κ -caseins in the humectant systems by lactocepin I from *Lactococcus lactis* subsp. *lactis* BN1 were compared. It should be noted that of the nine amino acid residues involved in substrate binding in lactocepin from strain BN1, just one is different from the corresponding residue in lactocepin I from *Lactococcus lactis* subsp. *cremoris* HP (Asp-142 in the strain HP enzyme [4] is replaced by an alanyl residue in the strain BN1 enzyme [29]). The lactocepin from strain BN1 does not fit precisely in any of the seven groups proposed by Exterkate et al. (4), but is nevertheless very closely related to the group containing the HP enzyme. In view of this and because its specificity with respect to casein hydrolysis is virtually identical to that of lactocepin I

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TABLE 1. NaCl and humectant concentrations in casein digests

Humectant	Humectant concn (% wt/vol)	NaCl concn (%, wt/vol)	a_w
PEG 20000	20.5	5	0.95
Glycerol	7.5	5	0.95
PEG 300	14	5	0.95
PEG 20000	20.5		0.99
Glycerol	7.5		0.98
PEG 300	14		0.99
None		5	0.97

from strain HP (unpublished data), the BN1 enzyme is referred to as lactocepin I below.

MATERIALS AND METHODS

Organism, growth conditions, and harvesting. *L. lactis* subsp. *lactis* BN1 was obtained from the culture collection of the New Zealand Dairy Research Institute, Palmerston North, New Zealand. Cultures were grown, harvested, and washed as described by Coolbear et al. (1).

Purification of lactocepin. Proteinase was released from the surface of washed lactococcal cells by using calcium-free phosphate buffer and was purified by using a combination of anion-exchange chromatography and gel exclusion chromatography (Mono Q HR 10/10 and Superose 6 HR 10/30 columns, respectively; Pharmacia Biotech, Uppsala, Sweden) as described by Coolbear et al. (1).

a_w measurements and humectants. All a_w measurements were made by using a model CX-2 dew point electronic humidity meter (Decagon Devices Inc., Pullman, Wash.). The humectants used were glycerol and polyethylene glycol 20000 (PEG 20000) (both from BDH Ltd., Poole, England), as well as PEG 300 (Merck, Darmstadt, Germany). The a_w measurements from a series of solutions of each humectant covering a range of concentrations in the presence and absence of 5% (wt/vol) NaCl were used to construct standard curves relating humectant concentration to a_w . Stock solutions of each humectant were made in 20 mM bis-Tris-propane (BTP) and adjusted to pH 6.4.

Casein substrates. κ -Casein (A variant) was a gift from K. Coolbear, Massey University, Palmerston North, New Zealand. β -Casein was a gift from R. Burr, New Zealand Dairy Research Institute, Palmerston North, New Zealand, and α_{s1} -casein was obtained from Sigma Chemical Co., St. Louis, Mo. Stock solutions of κ - and β -caseins (15 mg/ml) were made in 20 mM BTP (pH 6.4) while α_{s1} -casein was dissolved (15 mg/ml) in water.

Hydrolysis of casein. Casein samples (205 μ l) were hydrolyzed at 25°C with purified BN1 lactocepin I (17 μ l; 0.4 mg of protein/ml) in the presence of either humectant (470 μ l of stock solution) or, in the case of controls, 20 mM BTP (470 μ l) (Table 1). Samples (125 μ l) for high-performance liquid chromatography (HPLC) analysis were taken at 10 min, 30 min, 3 h, and 10 h, and each reaction was stopped by adding trifluoroacetic acid to a final concentration of 1% (vol/vol). Insoluble material was removed by centrifugation. Samples (25 μ l) for analysis by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were taken at the same times, and each reaction was stopped by boiling the reaction mixture with 25 μ l of 0.125 M Tris-HCl (pH 6.8) containing 4% (wt/vol) SDS, 20% (vol/vol) glycerol, and 10% (vol/vol) β -mercaptoethanol.

SDS-PAGE analysis. The presence of PEG 20000 in hydrolysate samples affected the resolution of protein bands obtained from the SDS-PAGE analysis (presumably due to the tendency of PEGs with molecular weights greater than 4,000 to bind weakly to SDS and migrate in a size-dependent manner during electrophoresis [31]). This problem was largely overcome by reducing the concentration of SDS in the electrode buffer from 0.1 to 0.05% (wt/vol). However, to obtain comparable resolutions for all samples, 10 μ l of 50% (wt/vol) PEG 20000 was added to all samples used for SDS-PAGE which did not otherwise contain PEG 20000, which resulted in the same final concentration of PEG 20000 in all cases. Electrophoresis was performed by using 15% (wt/vol) acrylamide gels and a model SE245 Mighty Small vertical gel apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). The sample loads were 10 and 8 μ l for samples with and without additional PEG 20000, respectively. The procedures used for preparation, electrophoresis, and staining were in general the procedures of Laemmli (13), as described by Reid et al. (22).

HPLC analysis. The presence of PEG 20000 in hydrolysate samples also affected the resolution of peaks obtained by HPLC analysis (improved resolution was obtained in some cases). As in the SDS-PAGE analyses, the concentrations of PEG 20000 in all samples were equalized by adding 40 μ l of 50% (wt/vol) PEG 20000 per 110 μ l of sample, as required. This also ensured that any effect of PEG 20000 on solubility of peptides was the same for all samples. Samples were analyzed as described by Reid et al. (22) by reverse-phase HPLC by using a Hewlett-Packard series 1050 HPLC equipped with a diode array detector, ChemStation software (Hewlett-Packard, Waldbronn, Germany), and a Vydac type 218TP C₁₈ column (Alltech Associates, Deerfield, Ill.). The injection volumes

used were 30 and 22 μ l for samples with and without additional PEG 20000, respectively.

Peptide identification. Peptides were identified as described previously (22) by performing an N-terminal sequence analysis with a model 476A protein sequencer (Applied Biosystems, Foster City, Calif.) and by determining molar masses by fast atom bombardment mass spectrometry by using a model VG-250 double-focusing magnetic sector mass spectrometer (VG Analytical, Manchester, United Kingdom) fitted with a cesium ion gun. Peptides were analyzed in a matrix of acidified glycerol.

RESULTS

The a_w of solutions containing PEG 20000, PEG 300, and glycerol at different concentrations in the presence of 5% (wt/vol) NaCl (i.e., the average salt-in-moisture content of premium grade New Zealand cheddar cheese [6]) were determined (Fig. 1) in order to obtain the humectant concentrations required in the presence of NaCl to give an a_w of 0.95. The a_w of humectant solutions at the same concentrations, but in the absence of NaCl, were also determined (Fig. 1). These concentrations of humectants (with and without 5% [wt/vol] NaCl) were used in all subsequent experiments. It was not possible to use PEG 20000 alone at an a_w of 0.95 as the concentration required resulted in precipitation of both substrate and enzyme.

SDS-PAGE analysis. (i) α_{s1} -Casein hydrolysis. In the presence of PEG 20000 alone, α_{s1} -casein was extensively hydrolyzed after 3 h of incubation with lactocepin I (Fig. 2a). A similar band pattern was obtained in the presence of PEG 20000-NaCl, although the extent of hydrolysis was clearly reduced. In the presence of the other humectants only very limited hydrolysis of α_{s1} -casein occurred, and the band patterns were very similar to the band patterns obtained in the control digests. The presence of NaCl further reduced what little hydrolysis was observed.

(ii) β -Casein hydrolysis. In the case of β -casein hydrolysis by lactocepin I (Fig. 2b), the fragment comprising residues 1 through 166 (fragment 1-166) rapidly accumulated in control digests and, to a lesser extent, in the presence of NaCl alone. This product was also the dominant product in the presence of glycerol or PEG 300 alone, but in the presence of either of these two humectants and NaCl the intensity of the band corresponding to fragment 1-166 was reduced, while the intensities of the lower-molecular-weight product bands were increased. Only very low amounts of fragment 1-166 were detected in hydrolysates obtained in the presence of PEG 20000

FIG. 1. Effect of humectant concentration (in the presence and absence of NaCl) on a_w . Symbols: ●, PEG 20000-NaCl; ○, PEG 20000; ▲, glycerol-NaCl; △, glycerol; ■, PEG 300-NaCl; □, PEG 300. The concentrations of the humectants (with and without 5% [wt/vol] NaCl) corresponding to an a_w of 0.95 are indicated by the dashed lines and are listed in Table 1.

FIG. 2. SDS-PAGE results, showing hydrolysis of α_{s1} -casein (a), β -casein (b), and κ -casein (c) by lactocepin I from *L. lactis* subsp. *lactis* BN1 under different a_w conditions. Hydrolysis was performed in the presence of no humectant, (control) (lanes 1), PEG 20000-NaCl ($a_w = 0.95$) (lanes 2), PEG 20000 ($a_w = 0.99$) (lanes 3), glycerol-NaCl ($a_w = 0.95$) (lanes 4), glycerol ($a_w = 0.98$) (lanes 5), PEG 300-NaCl ($a_w = 0.95$) (lanes 6), PEG 300 ($a_w = 0.99$) (lanes 7), and NaCl ($a_w = 0.97$) (lanes 8). For the concentrations of humectants and NaCl used, see Table 1. The digest times were as follows: for α_{s1} -casein all samples were taken at 3 h; β -casein samples were taken at 30 min (lanes 1 through 3, 5, 7, and 8) and 3 h (lanes 4 and 6); κ -casein samples were taken at 30 min (lanes 2, 3, 6 and 7) and 3 h (lanes 1, 4, 5 and 8). Bands corresponding to undigested substrate are indicated (α_{s1} -CN, β -CN, κ -CN), as are hydrolysis products from β - and κ -caseins {fragment (f) 1-166 [f(1-166)], f(1-160), f(1-95)} (see the text). The asterisks indicate the positions of two unique products.

alone, and no fragment 1-166 was detected with this humectant and NaCl.

(iii) κ -Casein hydrolysis. Marked differences in the rates of hydrolysis of κ -casein were found in the different humectant systems, with the most rapid hydrolysis occurring in the presence of PEG 20000 and PEG 300. The extent of κ -casein breakdown was therefore analyzed in these two systems after 30 min of hydrolysis, while the extent of κ -casein breakdown in all other systems was analyzed after 3 h (Fig. 2c).

The major hydrolysis product in all systems appeared to be

either fragment 1-160 or fragment 1-95 of κ -casein (identified by comparison with the data of Visser et al. [28]). The SDS-PAGE profiles obtained for hydrolysates in the control incubation mixtures and the profiles obtained in the presence of NaCl alone, glycerol alone, or PEG 300 alone (Fig. 2c) were similar, except for slightly lower levels of fragment 1-160 in the control. The SDS-PAGE profiles obtained for hydrolysates from incubation mixtures containing glycerol-NaCl or PEG 300-NaCl revealed significantly higher levels of fragment 1-160. The major differences in band patterns were seen in hydrolysates from preparations incubated in the presence of PEG 20000. In the presence of PEG 20000 alone, extensive hydrolysis occurred and apparently little fragment 1-160 remained, while in the presence of PEG 20000-NaCl the dominant product was fragment 1-95 and two unique product bands were also observed (Fig. 2c).

HPLC analysis. HPLC profiles of the trifluoroacetic acid-soluble peptides present in digests of α_{s1} -, β -, and κ -caseins are shown in Fig. 3 through 5, respectively.

(i) α_{s1} -Casein. Analysis of the α_{s1} -casein digest samples taken after 3 h of hydrolysis revealed only small quantities of peptides (Fig. 3). However, as anticipated from the SDS-PAGE analysis of corresponding samples, the greatest quantity of peptides was detected in the sample digested in the presence of PEG 20000 alone (Fig. 3, chromatogram c). With the exception of the digest containing PEG 20000, inclusion of NaCl appeared to suppress markedly the formation of low- M_r peptides.

(ii) β -Casein. The presence of NaCl, either alone or in the presence of glycerol, tended to suppress the formation of low- M_r peptides during hydrolysis of β -casein. However, the levels of the different peptides relative to each other were dependent on the humectant (Fig. 4). In the control the major peptides were the peptides comprising residues 164 to 168, 176 to 182, 166 to 175, 167 to 175, 169 to 175, 183 to 190, 194 to 209, and 73 to 93, while peptides comprising residues 1 to 6 and 47 to 52 were barely detectable. Conversely, the major peptides formed in the presence of PEG 20000, with and without NaCl (Fig. 4, chromatograms b and c, respectively), comprised residues 1 to 6 and 47 to 52, while the other peptides were present only at low levels. In the presence of PEG 300, with and without NaCl (Fig. 4, chromatograms f and g, respectively), hydrolysis gave moderate levels of peptides 1-6 and 47-52, but there were also moderate levels of the other peptides. The hydrolysate obtained from incubation in the presence of glycerol alone (Fig. 4, chromatogram e) gave a profile very similar to that of the control, with the exception that the levels of peptides 1-6 and 47-52 were high.

(iii) κ -Casein. Again, the presence of NaCl reduced the levels of the low- M_r peptides produced, particularly when NaCl was used alone (Fig. 5, chromatogram h) or in the presence of glycerol (Fig. 5, chromatogram d). Three of the major peptides produced by hydrolysis in the presence of PEG 20000-NaCl (comprising residues 96 to 100, 96 to 102, and 72 to 79) were also present in the control sample. However, the major peptide product under these conditions, comprising residues 152 to 160, was not detected either in the control sample or in samples from any of the other humectant systems. The converse was true for the peptide comprising residues 33 to 41, which eluted in a similar position.

DISCUSSION

No completely inert humectant (i.e., a humectant free of side effects, such as increased ionic strength, decreased surface tension, etc. [7]) is available. Therefore, distinguishing between

FIG. 3. HPLC chromatograms of the 1% trifluoroacetic acid-soluble peptides resulting from 3 h of hydrolysis of α_{s1} -casein under different a_w conditions by lactocepin I from *L. lactis* subsp. *lactis* BN1. Hydrolysis was performed in the presence of no humectant (control) (chromatogram a), PEG 20000-NaCl ($a_w = 0.95$) (chromatogram b), PEG 20000 ($a_w = 0.99$) (chromatogram c), glycerol-NaCl ($a_w = 0.95$) (chromatogram d), glycerol ($a_w = 0.98$) (chromatogram e), PEG 300-NaCl ($a_w = 0.95$) (chromatogram f), PEG 300 ($a_w = 0.99$) (chromatogram g), and NaCl ($a_w = 0.97$) (chromatogram h). For the concentrations of humectants and NaCl, see Table 1.

changes in enzyme activity related to a_w and changes in enzyme activity related to side effects is difficult. In a study of protease activity in the presence of various a_w depressors, Hertmanni et al. (9) found that changes in enzyme activity were humectant dependent, and they attributed the specificity changes ob-

served to an increase in medium hydrophobicity caused by water-structuring effects of the humectants. In the present study, three different humectants in combination with NaCl were used to mimic the a_w and salt content measured in cheddar cheese in order to identify effects on lactocepin specificity

FIG. 4. HPLC chromatograms of the 1% trifluoroacetic acid-soluble peptides resulting from 10 h of hydrolysis of β -casein under different a_w conditions by lactocepin I from *L. lactis* subsp. *lactis* BN1. For the conditions under which samples giving HPLC chromatograms a through h were hydrolyzed, see the legend to Fig. 3. The peptides identified in the major peaks labelled peaks 1 through 10 comprised, respectively, residues 164 to 168; 176 to 182; 166 to 175, 167 to 175, and 169 to 175; 1 to 6 and 47 to 52; 183 to 190; 142 to 146; 57 to 72; 57 to 68; 194 to 209; and 73 to 93.

FIG. 5. HPLC chromatograms of the 1% trifluoroacetic acid-soluble peptides resulting from 10 h of hydrolysis of κ -casein under different a_w conditions by lactocepin I from *L. lactis* subsp. *lactis* BN1. For the conditions under which samples giving HPLC chromatograms a through h were hydrolyzed, see the legend to Fig. 3. The peptides identified in the major peaks labelled 1 through 6 comprised, respectively, residues 96 to 100, 96 to 102, 66 to 71, 152 to 160, 33 to 41, and 72 to 79. The N-terminal residue of the peptide identified in peak 7 corresponded with residue 107 of κ -casein, but its C-terminal residue was not determined.

unique to particular humectants. Furthermore, PEGs having very different chain lengths (and therefore different relative hydrophobicities) were used in order to determine any changes in enzyme activity or specificity that depended on hydrophobicity effects. In addition, the use of different humectants ensured that changes in solubility of intermediate peptides formed during the enzyme reaction (and therefore their potential removal from the enzyme system) could be recognized. The lactocepin used in the present study, the lactocepin from *L. lactis* subsp. *lactis* BN1, was known to possess type P₁ activity.

A comparison of the specificity of hydrolysis of β - and κ -caseins obtained by using lactocepin I in buffer alone (i.e., the control) with the specificity of hydrolysis obtained with each of the low- a_w systems revealed some significant differences. In these systems β -casein fragment 1-166 (which is diagnostic for lactocepin I in buffer alone) was not produced, β -casein peptide 47-52 and κ -casein peptide 152-160 were formed, and there were elevated levels of κ -casein fragment 1-160. β -Casein peptide 47-52 and the two κ -casein peptides are typical products of lactocepin III activity in buffer alone (21, 23, 26, 28). In fact, it has been suggested that hydrolysis of the κ -casein Glu-151-Val-152 bond to give the peptide 152-160 is a feature that characterizes lactocepin III action on κ -casein (28) in buffer alone, as does production of κ -casein fragment 1-160 (21). The overall effect of the various combinations of humectants was, therefore, to impart some lactocepin III specificity characteristics to lactocepin I.

Elevated levels of κ -casein fragment 1-160 appeared to result from reduced a_w , as this effect was most prominent in the systems containing PEG 300-NaCl or glycerol-NaCl, both of which had an a_w of 0.95. Although increased levels of this fragment (relative to the control) were also detected in the presence of NaCl alone, PEG 300 alone, or glycerol alone when the a_w was higher (range, 0.97 to 0.99), the effect in these systems was less marked. Similarly, the elevated levels of β -ca-

sein peptide 47-52 obtained in the presence of PEG 300-NaCl were also obtained in the presence of PEG 300 alone and glycerol alone (a_w , 0.99 and 0.98, respectively) and therefore may have been dependent on reduced a_w , even when the reduction was only moderate. However, many of the differences observed in the presence of PEG 20000-NaCl (a_w , 0.95) were also observed in the presence of PEG 20000 alone (a_w , 0.99), and some differences, such as production of κ -casein peptide 152-160, occurred only when PEG 20000 was used as the humectant. Furthermore, hydrolysis of α_{s1} -casein was extensive in the presence of PEG 20000 alone, whereas at the same a_w hydrolysis was minimal in the presence of PEG 300 alone.

These findings show that many of the specificity changes resulted from factors other than reduced a_w . It has been suggested that PEG may bind to an enzyme, thereby increasing its microenvironmental hydrophobicity and decreasing the K_m for a hydrophobic substrate, an effect which is dependent on the molecular weight (and therefore hydrophobicity) of the PEG (11). In the present study, therefore, a combination of reduced a_w and the hydrophobicity of PEG 20000 most probably played a significant role in altering the specificity of lactocepin I (by inference, the hydrophobicity index of PEG 20000 is at least 34-fold greater than that of PEG 300 [11]). The presence of PEG may also have imposed a more ordered structure on the caseins, resulting in some of the observed changes in specificity.

The finding that the reduced a_w generated by glycerol affected specificity to a lesser extent than the a_w generated by the PEG humectants may be related to the ability of glycerol to form hydrogen bonds with functional groups of protein molecules, which under low- a_w conditions may otherwise interact with each other (30). Therefore, the effect of glycerol (like that of water) may have been to "unlock" the protein structure (of either enzyme or substrate) and promote activity.

Changes in a_w may also modify enzyme behavior either directly by changing the ionization state of the enzyme or indi-

rectly by changing the conformation of the substrate (18). In the latter case previously inaccessible regions of the polypeptide chain may be exposed, leading to hydrolysis at new sites. In water, an important driving force for substrate binding is the hydrophobic effect; a reduction in a_w (i.e., a reduction in the free water content of the medium) may lead to subtle changes in binding forces, thereby altering the enzyme's preference for certain binding sites and, therefore, enzyme specificity.

An important consideration in cheese itself is the continuous change in the a_w of cheese during manufacturing and ripening which results from water loss, salt diffusion (mainly in brine-salted cheeses), substrate-salt interactions, and the accumulation of low- M_r water-soluble compounds. Furthermore, at any given time during ripening, an array of compounds contributes to the lowered a_w of cheese, and each of these, like any other humectant, may give rise to specific side effects which have an impact on enzyme action. As the overall composition of these humectant-like compounds changes during ripening, so too do the associated side effects. There are additional factors to take into account. For example, Exterkate and Altling (3) showed that the specificities of two cell-bound lactocepins differed somewhat from those of their soluble counterparts at different pH values. This may also be the case for different forms of lactocepin with respect to the cheese environment. Whether lactocepin remains cell associated during cheese ripening is unknown. However, Coolbear et al. (1) showed that disruption of the cell wall structure results in release of lactocepin from the cell, even in the presence of up to 50 mM Ca^{2+} ; as starter cells die off early in the ripening process, it is likely that the integrity of the cell wall structure is compromised. This is likely therefore to lead to the release of soluble lactocepin into the cheese matrix. The location of starter cells in the cheese matrix may also be significant, as the results of a recent study indicate that from a very early stage in the ripening of cheddar cheese, the cells appear to be in direct contact with the fat globule membrane or are located at the casein-fat interface (14). Based on results of the present study, the location of lactocepin in such a hydrophobic microenvironment very likely affects its activity and specificity.

In summary, the low- a_w -high-salt systems clearly showed that the specificity of lactocepin is dependent on a_w and medium hydrophobicity. Previous work has shown the potential effects of salt and pH on specificity (3, 5). It is clear that the characteristic differences of the lactocepin types in buffer alone are not fully reflected in the cheese environment. In order to gain a more accurate understanding of the impact of the different lactocepins on cheese quality and flavor, the complex effects of the cheese environment on the specificities of these enzymes, and indeed the whole proteolytic system, have to be carefully considered.

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