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MINIREVIEW

Casein Utilization by Lactococci

EDDY J. SMID,† BERT POOLMAN,* AND WIL N. KONINGS

Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

INTRODUCTION

Mixed starter cultures used to manufacture Dutch cheeses include *Lactococcus* and *Leuconostoc* species, the former being quantitatively more important (3, 13). Several metabolic properties of lactococci serve special functions which directly or indirectly have an impact on processes such as flavor development and ripening of the cheese (31). These functions are (i) fermentation and depletion of the milk sugar lactose, (ii) reduction of the redox potential of the cheese, (iii) citrate fermentation, and (iv) casein degradation. Casein degradation by lactococci yields free amino acids, as well as low- and high-molecular-weight peptides. These are the sources of essential and growth-stimulating amino acids for the members of the genus *Lactococcus* (42). Besides being an important nutritional source for the starter bacteria, casein also plays a crucial role in the development of flavor in cheese. Certain peptides contribute to the formation of a typical cheese flavor, and other, undesirable bitter-tasting peptides can be produced (39, 45, 47). A better understanding of the processes leading to the formation of these flavor peptides requires knowledge about all enzymes involved in casein degradation.

The sequential steps involved in β-casein degradation and the cellular location of the enzymes involved will be reviewed in this paper. Special attention will be paid to the role of amino acid and peptide transport systems in the overall process of casein degradation and the overall growth performance of the organism. On the basis of the proteolytic breakdown products of casein and the substrate specificities of the peptidases and transport systems, a minimum model for the growth of lactococci in casein-containing media is proposed.

THE PROTEOLYTIC SYSTEM

Caseins constitute about 80% of all proteins present in bovine milk (total protein content, 3.0 to 3.5% [wt/wt]) and serve as the major organic nitrogen source for starter culture growth in milk fermentations (11, 26, 41). The four different types of casein species found in milk, α_{s1}-, α_{s2}-, β-, and κ-caseins, are organized in micelles to form soluble complexes (34). For cheese manufacturing, a starter culture, together with rennet (containing the enzymes chymosin and bovine pepsin), is added to the milk. The chymosin acts on κ-casein, which is present at the surface of casein micelles. As a result, casein micelles coagulate, and α_{s1}-, α_{s2}-, and β-caseins become exposed and can be hydrolyzed by the lactococcal caseino- and peptidolytic enzymes.

* Corresponding author.
† Present address: ATO Agrotechnological Research Institute, Haagsteeg 6, 6700 AA Wageningen, The Netherlands.

The enzymes which are active in casein degradation by lactococci are (i) a cell wall-associated caseinolytic proteinase, (ii) an extracellular peptidase(s), (iii) amino acid transport systems, (iv) peptide transport systems, and (v) intracellular peptidases. All these enzymes together form the proteolytic system of lactococci. Recently, the genetics (22) and the biochemistry (24) of the proteolytic system were reviewed.

GROWTH ON CASEIN-CONTAINING MEDIA

The specific rates of growth of several strains of *Lactococcus lactis* in milk are 10 to 40% lower than those in broth media or in amino acid-containing chemically defined media (14, 16, 38). Growth studies with milk containing ^{14}C-labelled milk protein have shown that the caseins become more important relative to free amino acids in supplying nitrogen as the cell density increases (26). Hugenholtz et al. (14) have shown that the specific rate of growth of *L. lactis* subsp. *cremoris* Wg2 in milk is determined by the rate of casein hydrolysis and that casein limitation leads to a limitation of amino acid supply. Whether the casein-derived amino acids were supplied to the cells as free amino acids or as small peptides remained unanswered. On the basis of casein hydrolysis studies with several *Lactococcus* strains, β-casein seems to be the most readily utilized milk protein (9, 12, 15). Growth studies with *L. lactis* subsp. *cremoris* HP in chemically defined media supplemented with different caseins alone and in combinations (as the sole source of organic nitrogen) revealed that β-casein in combination with a relatively low concentration of κ-casein supported maximal growth (11). Therefore, the soluble β-casein fraction, which is in equilibrium with micellar β-casein (5), and the easily accessible hydrophilic part of micellar κ-casein are most likely the major sources of essential and growth-stimulating amino acids for lactococci (11).

PROTEINASE SPECIFICITY

The cell envelope-attached caseinolytic proteinase is the first enzyme in the pathway of casein degradation (21). On the basis of the patterns of breakdown of α_{s1}-, β-, and κ-caseins, two specificity classes could be discriminated (44). These activities are generally referred to as P_I (reference strain HP) and P_{III} (reference strain AM_1). The primary substrate of P_I-type enzymes is β-casein, while P_{III}-type enzymes degrade both α_{s1}- and β-caseins. Although both types of enzymes attack β-casein, the casein degradation patterns and thus the specificities differ. Moreover, several strains exhibit a mixed P_I-P_{III}-type specificity (7, 44) which appears to be the result of a single gene product (21). Proteinase genes of each specificity class have been cloned and sequenced (22, 48). Recently, hybrid proteinase genes

53-68
ala-gln-thr-gln-ser-leu-val-tyr-pro-phe-pro-gly-pro-ile-pro-asn
        thr-gln-ser-leu
                ser-leu-val-tyr-pro-phe-pro-gly-pro-ile-pro-asn
                leu-val-tyr-pro-phe-pro-gly-pro-ile-pro-asn

69-93
ser-leu-pro-gln-asn-ile-pro-pro-leu-thr-gln-thr-pro-val-val-val-pro-pro-phe-leu-gln-pro-glu-val-met

164-175
ser-leu-ser-gln-ser-lys-val-leu-pro-val-pro-gln
ser-leu-ser-gln-ser
        gln-ser-lys-val-leu-pro-val-pro-gln
        lys-val-leu-pro-val-pro-gln

176-182
lys-ala-val-pro-tyr-pro-gln

183-191
arg-asp-met-pro-ile-gln-ala-phe-leu
arg-asp-met-pro-ile-gln-ala-phe

194-209
gln-gln-pro-val-leu-gly-pro-val-arg-gly-pro-phe-pro-ile-ile-val

FIG. 1. Amino acid sequences of the oligopeptides released from β-casein by the proteinase of *L. lactis* subsp. *cremoris* AC1. The numbers correspond to the amino acid residues in β-casein. Data were taken from reference 27.

were constructed (21), and the caseinolytic activities were compared. At most, seven amino acid substitutions in the N-terminal region and two in the C-terminal region were found to be responsible for the different types of activity (21).

The action and product formation of purified P_I proteinases from three different *L. lactis* strains on β-casein have been studied in great detail (27, 28, 46). The peptides released by the proteinases were identified. They all originated from two areas of the β-casein sequence (between amino acids 53 to 93 and the C-terminal amino acids 129 to 209). In particular, the C-terminal hydrophobic fragment {fragment from amino acid residues 194 to 209 [f(194-209)], Fig. 1} has been identified as a major source of bitter-tasting peptides (47).

The peptides released by the proteinase from *L. lactis* AC1 are shown in Fig. 1 and 2. They have an average size of 11 to 12 amino acid residues. The proline content of β-casein is 16.7% (35 proline residues per 209 residues). The proline contents of the identified peptides are even higher (21.2, 21.6, and 23.4% for strains HP, NCDO 763, and AC1, respectively).

The peptides liberated by the proteinases are, with a few exceptions, too long to be taken up by *L. lactis* (Fig. 2). Some oligopeptides with up to 6 amino acid residues can most likely be taken up directly. However, tetrapeptides containing prolyl, glutamyl, aspartyl, or arginyl residues are not utilized at all (35). Apparently, only dipolar ionic oligopeptides can be transported via the lactococcal oligopeptide transport system. Considering the substrate specificity of the oligopeptide transport system, the β-casein-derived peptides f(55-58) and f(164-168) can be taken up without additional hydrolysis (Fig. 2). However, these peptides do not contain all the essential amino acids needed to satisfy the lactococcal nutritional demands (35). Furthermore, tyrosine is the only free amino acid identified in the β-casein hydrolysate.

EXTRACELLULAR OLIGOPEPTIDE HYDROLYSIS

The oligopeptides derived from casein (Fig. 1) must be hydrolyzed further by extracellular peptidases to obtain peptides which can be translocated into the cells by the lactococcal peptide transport systems. These (amino) peptidases also produce free amino acids which can be taken up by the cells via the different specific amino acid transport systems (Table 1; see Konings et al. [23] for details).

It is now well established that several different peptidase activities are present in a single *Lactococcus* strain (19). To understand how the lactococci obtain their essential and growth-stimulating amino acids from β-casein degradation, it is of primary importance to establish which of the lactococcal peptidases contribute to the extracellular hydrolysis of the oligopeptides produced by the caseinolytic proteinase. The cellular locations of the peptidases have not been clearly established. Often, the same peptidase can be isolated from a culture supernatant and from a cell extract. In addition, a number of these peptidases also appear to be associated with the cell envelope (8, 39a).

Since carboxypeptidase activity has not been identified in *Lactococcus* strains, apparently these organisms can only degrade the casein-derived peptides from the N terminus. Geis et al. (12) described the purification of an aminopeptidase from *L. lactis* subsp. *cremoris* AC1. This peptidase was isolated with the gentle washing procedure described for the extraction of the cell envelope-attached proteinase. Since this procedure keeps the bacterial cells intact (12), the aminopeptidase is most likely active outside the cells and is therefore accessible to peptides released from casein by the proteinase. Also, the aminopeptidase described by Tan and Konings (40) could be extracted from intact cells of *L. lactis* supsp. *cremoris* Wg2 simply by washing. This procedure did not result in the release of intracellular marker enzymes, indicating that this aminopeptidase also can be found outside the cells. On the other hand, the aminopeptidase isolated from *L. lactis* subsp. *cremoris* AM2 is most likely located in the cytoplasm (30). These observations suggest that at least
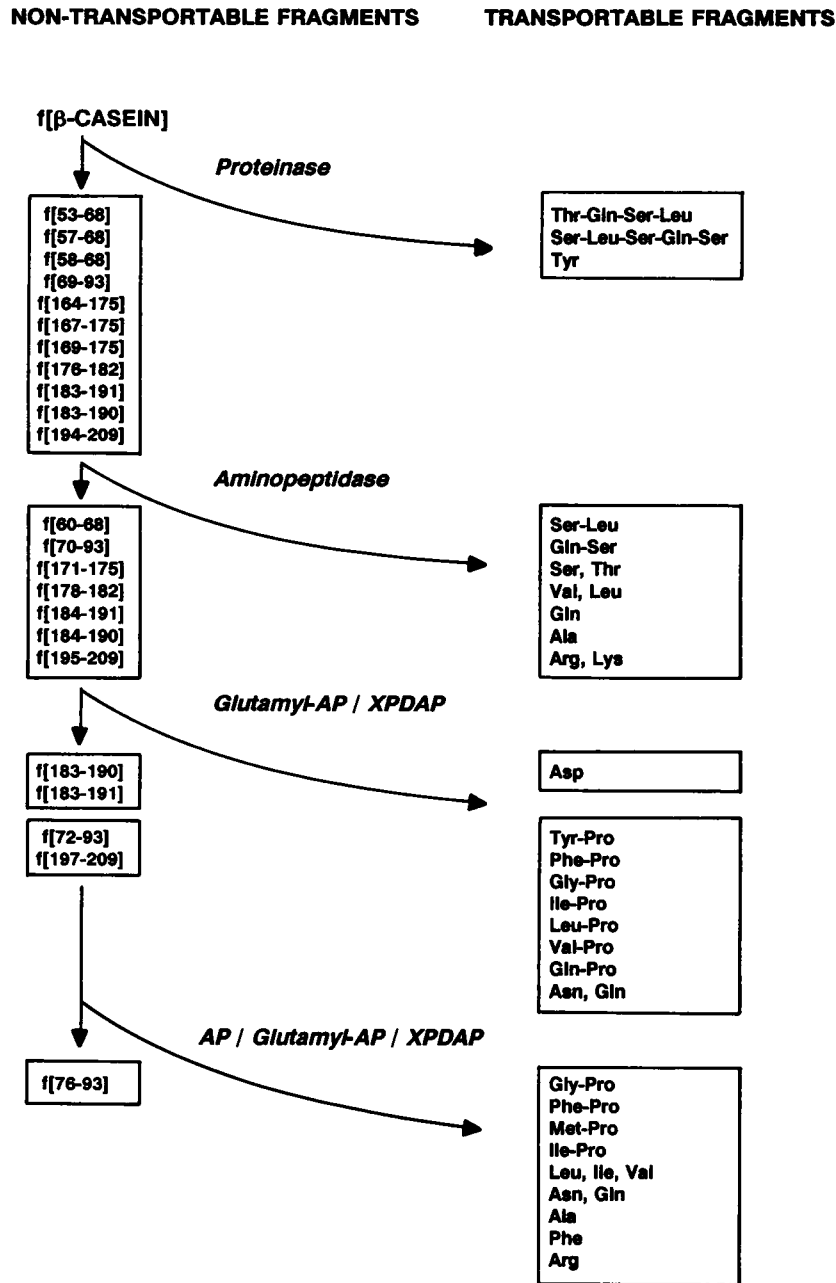
NON-TRANSPORTABLE FRAGMENTS   TRANSPORTABLE FRAGMENTS

FIG. 2. Peptide fragments and amino acids derived from β-casein by the subsequent action of extracellularly located proteinase, aminopeptidase (AP), X-PDAP, and glutamyl aminopeptidase (glutamyl-AP). The "non-transportable fragments" derived by proteinase action correspond to those of strain AC1 (Fig. 1).

one general aminopeptidase contributes to the extracellular degradation of peptides derived from β-casein (Fig. 2). On the basis of the available information on substrate specificities of aminopeptidases (12, 20, 40), it is possible to predict which free amino acids and peptides will be released from the peptides derived from casein. Aminopeptidase activity on the peptides derived from β-casein (Fig. 1) will lead to the production of serine (9), threonine (2), glutamine (6), valine (6), leucine (5), alanine (2), arginine (2), and lysine (4) (Fig. 2). Furthermore, the dipeptides Ser-Leu and Gln-Ser will be produced from f(55-58) and f(164-168), respectively. These

amino acids and dipeptides can be taken up by the cells via amino acid transport systems and di- and tripeptide transport systems. However, this mixture of amino acids does not include isoleucine, methionine, histidine (essential for all lactococci), and proline and phenylalanine (essential for stimulation of growth of lactococci) and thus cannot support the growth of *L. lactis* (32, 33).

Peptide f(60-68) contains the full sequence of β-casomorphin, an oligopeptide which has been shown to be an excellent substrate for the recently described X-prolyl-dipeptidyl aminopeptidase (X-PDAP) from *L. lactis* subsp.

TABLE 1. Mechanism of energy coupling and specificity of amino acid and peptide transport systems of lactococci[a]

Transport system	Mechanism[b]	Specificity
Branched-chain amino acid	PMF	Leu, Ile, Val
Neutral amino acid I	PMF	Ala, Gly
Neutral amino acid II	PMF	Ser, Thr
Glutamate	FP	Glu, Gln
Asparagine	FP	Asn
Aspartate	Exchange	Asp, Glu[c]
Basic amino acid I	PMF	Lys, Orn
Basic amino acid II	PMF	His[d]
Arg-Orn antiporter	Exchange	Arg, Orn, Lys
Aromatic amino acid	PMF	Phe, Tyr, Trp[e]
"Proline"	Diffusion	
Di- and tripeptides	PMF	Di- and tripeptides[f]
Oligopeptide	Unknown	Tri and hexapeptides[g]

[a] Details of the individual transport systems have been reviewed in reference 22.
[b] PMF, proton motive force; P, energy-rich phosphate bond.
[c] System with low affinity for acidic amino acids.
[d] Substrate specificity has not been studied.
[e] Substrate specificity has not been studied in detail.
[f] Arginine-containing peptides are not transported.
[g] Neutral oligopeptides not containing Pro residues.

cremoris (20). X-PDAP was isolated from the cell wall fraction (6, 12, 25), suggesting that the enzyme can be active at the outer surface of the cells. β-Casein-derived peptides f(60-68), f(171-175), and f(178-182) with the X-Pro-Y-Pro-Z motif can be degraded by this enzyme into several X-Pro dipeptides. In addition, hydrolysis of f(70-93) and f(195-209) can be initiated by this enzyme. Uptake of the f(60-68)-, f(171-175)-, and f(178-182)-derived X-Pro dipeptides supplies the cells with essential and/or growth-stimulating amino acids (proline, phenylalanine, and isoleucine).

The X-Pro dipeptides produced by X-PDAP were found to be high-affinity substrates for the lactococcal di- and tripeptide transport system (37). Since free proline can enter lactococcal cells only by passive diffusion across the cytoplasmic membrane (37), the importance of a functional di- and tripeptide transport system for the growth of *L. lactis* on media containing casein as a source of amino acids is clear (38).

Oligopeptides f(184-190) and f(184-191) have N-terminal aspartate residues. Most likely, a highly specific glutamyl-aminopeptidase (10) acts on these fragments. The enzyme was shown to be present in different *Lactococcus* strains (12a) and is possibly active at the outer surface of the cell membrane (8, 10). The released aspartate can be taken up by the cells via a specific transport system (Table 1) (23). Oligopeptides f(185-190) and f(185-191) can be degraded by the combined action of X-PDAP and a general aminopeptidase. The released dipeptide Met-Pro can be taken up via the lactococcal di- and tripeptide transport system, thereby finally supplying the cells with the essential amino acid methionine (Fig. 2).

The oligopeptides released from β-casein by AC1 and HP proteinases do not contain histidine (27, 46). Only oligopeptides f(129-141), f(131-141), and f(142-160), released by *L. lactis* subsp. *lactis* NCDO 763 proteinase, contain histidine residues (27). Since histidine is an essential amino acid for lactococci (32, 33, 35a), β-casein cannot be the only organic nitrogen source for some strains (i.e., HP and AC1). Exterkate and de Veer (11) have shown that β-casein in combination with a relatively low concentration of κ-casein is the best substrate for the growth of *L. lactis* subsp. *cremoris*

HP. A feasible explanation for this observation is that κ-casein supplies the cells with histidine.

INTRACELLULAR PEPTIDE HYDROLYSIS

During the process of extracellular β-casein degradation, several di-, tri-, and oligopeptides which can be taken up by the lactococci are released. After uptake, the peptide bonds are hydrolyzed and amino (and imino) acids are released (36). Hence, intracellular peptidases must be active in *L. lactis*.

Several peptidases isolated from crude cell extracts of *L. lactis* have been reported; these include dipeptidases (17, 43), tripeptidases (1, 2), aminopeptidases (4, 30), a prolidase (18), and endopeptidases (29, 49). With a few exceptions (17, 43), an intracellular location for these enzymes has been postulated (1, 4, 29, 30, 49) or suggested (2, 18).

It was demonstrated conclusively that intact di- and tripeptides are taken up by *L. lactis* and that the accumulation of free amino acids in intact cells occurs when transportable peptides are supplied (36). Obviously, some of the peptidases listed above are located and are active inside cells. An example is the prolidase described by Kaminogawa et al. (18). This enzyme hydrolyzes exclusively X-Pro dipeptides and thus releases free proline and the accompanying amino acid. Other di- and tripeptides translocated into cells can be hydrolyzed by a dipeptidase (43) and a tripeptidase (2), respectively.

THE BALANCED PROTEOLYTIC SYSTEM

The degradation of caseins is accomplished by a complex array of sequential steps in which peptide bonds are hydrolyzed and the products of hydrolysis are transported into the cells. On the basis of the presented analysis of β-casein utilization, at least four extracellular proteolytic and peptidolytic enzymes must be operative; these include (i) the caseinolytic proteinase, (ii) a general aminopeptidase, (iii) an X-PDAP, and (iv) a glutamyl-aminopeptidase (Fig. 2). The concerted action of these four enzymes could supply the cells with a complex mixture of amino acids, dipeptides, tripeptides, and oligopeptides which could be translocated into the cells via the known transport systems (Table 1). In this scheme, the essential or growth-stimulating amino acids isoleucine, methionine, phenylalanine, and proline would be supplied exclusively as X-Pro dipeptides.

The model presented does not take into account the possible involvement in casein degradation of an extracellularly located endopeptidase. Although different endopeptidases have been isolated and characterized (40a, 49), their cellular locations are still not clear. The purpose of this paper, however, was to review the enzymes involved in casein degradation and to present a minimal model on how lactococci can be supplied with their essential and growth-stimulating amino acids when growing on casein(s). Additional peptidase activities, e.g., an extracellular endopeptidase(s), may improve this model.

The optimal growth of *L. lactis* requires a balanced supply of different amino acids. A change in the activity of a single step of the proteolytic system can be reflected as a change in the specific bacterial growth rate. This is evident from the impact of the manipulation of dipeptide transport on the growth of *L. lactis* (37, 38). A heterogenous mixture of dipeptides must enter the cells via the di- and tripeptide transport system, and all these peptides compete for uptake via this system. The observation that even relatively low

concentrations of exogenously supplied dipeptides inhibit lactococcal growth severely (37) indicates that the composition of the mixture of casein-derived peptides is well balanced for rapid growth on casein. If this concept is correct, the specific overproduction of the extracellular proteinases or peptidases (by genetic manipulation) could result in growth inhibition due to an imbalance in the peptide supply.

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