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Review

Proteolytic System of *Streptococcus thermophilus*

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The growth of lactic acid bacteria (LAB) generates a high number of metabolites related to aromas and flavors in fermented dairy foods. These microbial proteases are involved in protein hydrolysis that produces necessary peptides for their growth and releases different molecules of interest, like bioactive peptides, during their activity. Each genus in particular has its own proteolytic system to hydrolyze the necessary proteins to meet its requirements. This review aims to highlight the differences between the proteolytic systems of S*treptococcus thermophilus* and other lactic acid bacteria (*Lactococcus* and *Lactobacillus*) since they are microorganisms that are frequently used in combination with other LAB in the elaboration of fermented dairy products. Based on genetic studies and in vitro and in vivo tests, the proteolytic system of *Streptococcus thermophilus* has been divided into three parts: 1) a serine proteinase linked to the cellular wall that is activated in the absence of glutamine and methionine; 2) the transport of peptides and oligopeptides, which are integrated in both the Dpp system and the Ami system, respectively; according to this, it is worth mentioning that the Ami system is able to transport peptides with up to 23 amino acids while the Opp system of *Lactococcus* or *Lactobacillus* transports chains with less than 13 amino acids; and finally, 3) peptide hydrolysis by intracellular peptidases, including a group of three exclusive of *S. thermophilus* capable of releasing either aromatic amino acids or peptides with aromatic amino acids.

Keywords: Proteolysis, lactic acid bacteria, *Streptococcus thermophilus*

Introduction

The proteolytic system of lactic acid bacteria (LAB) has been extensively studied using genetic and biochemical methods. Recently, research has been focused on the production of bioactive peptides by specific action of the proteolytic system over milk proteins during its fermentation [1, 2]. In addition, several studies have been carried out in order to prove that the proteolytic systems of lactococci and lactobacilli hold a close relation [3, 4], while the proteolytic system of *Streptococcus thermophilus* presents other characteristics that makes it necessary to study it separately. The sequence of proteolytic reactions that occurs during streptococci as well as for lactococci and lactobacilli

growth in milk is triggered by the action of extracellular endopeptidases. These proteinases release peptides derived from different caseins, which are natural substrate for intracellular peptidases that convert them into smaller peptides or, in case of an extent of hydrolysis, into amino acids. In general, the proteolytic system consists of serial chained reactions (Fig. 1), that are started by a serine proteinase proline-specific, linked to the cell wall; followed by the specific transport of dipeptides, tripeptides and oligopeptides, and finally, by the action of a countless number of intracellular peptidases and aminopeptidases [1, 5].

It has been clearly stated that many lactic acid bacteria are multi-auxotrophic for amino acids [6, 7]. These requirements
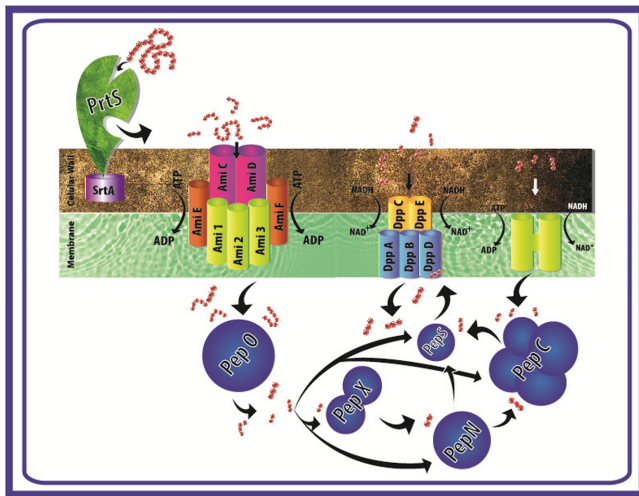
Fig. 1. Streptococcus thermophilus proteolytic system FOR.
Graphic presentation of the proposed proteolytic system of *Streptococcus thermophilus*, its function and regulation. Model follows that proposed by Kuji *et al.* (1996). The system is divided into three steps. The first step is the breakdown of proteins by a proteinase PrtS, which is anchored at cell wall by sortase A (SrtA). The second step is transport of peptides and oligopeptides, the transport of peptides is integrated by the Dpp system with five proteins (DppA, DppB, DppC, DppD, and DppE) regulated by ATP and proton formation (NAD+); the oligopeptides transport is carried out by Ami system integrated in a operon system whit seven proteins (Ami1, Ami2, Ami3, AmiC, AmiD, AmiE, and AmiF) regulated by ATP and activate by sulfur amino acid presence. The transcriptional regulatory protein ComR associated to Ami system is regulated by the peptide ComS. Finally, third step is the cut of peptides by fourteen intracellular peptidases, where five (PepO, PepS, PepX, PepN, and PepC) have different characteristics and specificity to those of other lactic acid bacteria.

are specific for each strain, for example, some strains of *Lactobacillus* need only 4 amino acids, while others like *Streptococcus thermophilus* require from 14 to 20 depending on the strain [3, 8]. The requirements for this bacterium are 4 times of glutamine and 14 times of methionine, in comparison to those reported for *Lactobacillus* spp. [8, 9]. On the other hand, *S. thermophilus* utilizes sulfured amino acids available in the medium to avoid energy consumption for their biosynthesis [10, 11].

Due to low activity of peptidases of *S. thermophilus*, the transport of amino acids and oligopeptides is more efficient than in other lactic acid bacteria, in which the intake of amino acids does not require energy consumption [12, 13]. Besides, *S. thermophilus* has a specific intracellular peptidase for aromatic amino acid, such as phenylalanine and tryptophan [14, 15]. The activity of this peptidase releases peptides with amino acids, which are essential

for the growth of *S. thermophilus*. Also, a link between *S. thermophilus* growth in milk with high acidifying capacity and the presence of an efficient proteolytic system has been demonstrated [16].

Cell Wall Proteinase of Lactic Acid Bacteria

The degradation of caseins by LAB is triggered by an extracellular proteinase linked to the cell wall called PrtP in lactococci and lactobacilli [1, 4, 17, 18]. In the case of *S. thermophilus*, the proteinase linked to the cell wall is called PrtS. This enzyme has specific differences with the cell wall proteinase of other lactic acid bacteria [19]. One of the most important differences is the isoelectric point (4.6) which is lower than the PrtP (4.8), that leads to the precipitation and subsequent inactivation of PrtS [20].

PrtP is classified according to its specificity in PI and PIII. It is a monomeric serine proteinase weighing between 180 and 190 kDa, although Laan and Konings [21] found some fractions associated with this proteinase during its isolation, suggesting that this fraction is monomeric only under certain conditions where the medium has neutral pH. The gene that encodes for PrtP has been cloned and sequenced from some LAB. Primary structure of this enzyme consists of around 1,902 amino acids for lactococci and 1,946 to 1,962 amino acids for lactobacilli species [3]. Proteinases of different *Lactococcus* species present a similarity of 98% while the similarity of proteinases from lactobacillus species is 95%, except the proteinase from *Lactobacillus delbrüeckii*, which presents a similarity of only 40% [22].

In a study on the proteolytic capacity of LAB, Shihata and Shah [23] observed that some streptococcal species showed higher proteolytic activity than other lactic acid bacteria, including lactobacilli and bifidobacteria. These results can be better explained with the requirements of glutamic acid and methionine, which are nearly five to six times higher for *Streptococci* than for the content of these amino acids released in milk (45 and <1 mg/ml, respectively) [24]. It is known that the *prtS* sequence encoding for the proteinase is similar to the sequences for other proteinases of lactic acid bacteria [25, 26].

PrtS is anchored to the cell wall of *S. thermophilus* by sortase A (SrtA) through a LPXTG sequence of the proteinase in the extreme C-terminal [27]. Likewise, it has been observed that this proteinase is necessary in the proto-cooperation systems like the yoghurt process [26]. Courtin *et al.* [26] tracked the changes in pH and the development of *S. thermophilus* and *L. bulgaricus* during milk fermentation and observed that the action of PrtS proteinase was essential for the growth of *S. thermophilus* in

a pure culture, but it did not have any effect on its growth in a mixed culture with *L. bulgaricus* due to the presence of the particular proteinase PrtB of lactobacillus and PrtP of lactococcus homologue. However, proteinase PrtB of *L. bulgaricus* became essential for the growth of *S. thermophilus* in mixed cultures where a higher decrease of pH was found in pure cultures.

Specificity of *Streptococcus thermophilus* Proteinase PrtS

The products derived from the action of proteinases in milk are mainly from caseins αS1 and β, which are the most abundant proteins. These proteins are preferred by the proteolytic system of LAB in over 80% of serum proteins [1, 5]. In the case of β-casein (BCN), some studies have revealed that the action of both PrtP and PrtS, can generate more than 100 oligopeptides with sizes from 4 to 30 residues, without any indications of the existence of di- and tri-peptides but with trace concentrations of phenylalanine. Many peptides are generated from region 60-105 of this protein [1, 22, 29]. It has been observed in recent studies, that residues generated by the hydrolysis of sodium caseinate by *S. thermophillus* 4F44 and *S. termophilus* LMD-9-Δ*srtA* PrtS, are peptides with different biological functions such as antihypertensive, anti-inflammatory, antioxidant, etc. [20, 30].

Regarding κ-casein (KCN), the oligopeptides are generated from the region 96-106 [3]. BCN and KCN can be degraded by both types of PrtP of lactococci, PI and PIII; nevertheless, in the case of α-casein (ACN) degradation can only occur through proteinase PIII and some proteinases with intermediate specificity between PI and PIII, since PII is unable to hydrolyze caseins α-S1 and α-S2. Nearly 23 oligopeptides coming from C-terminal end of ACN have been found [31].

In addition, specificity of PrtS of *S. thermophilus* is intermediate between that of PI and PIII of lactococcus. This enzyme has affinity to the three types of caseins [19, 32]. Fernandez-Espla *et al*. [19] observed that PrtS cuts links between an aromatic amino acid in position 1 (P1) and a proline in position 2 (P2), but becomes inactive when an amino acid is charged negatively in position 3 (P3). On the other hand, the sequence Ala-Pro-Tyr, that is preferred by PI for cutting, was the one with the highest activity for PrtS, whereas PIII, which shows more affinity for Ala-Glu-Pro-Tyr-Phe, was poorly degraded by PrtS. There is in general a similar degradation of total caseins and BCN (degradation BCN/degradation of caseins = 0.8), indicating that PIII PrtS does not have a marked preference for BCN. Also, depending on the conditions of reaction (pH, saline

concentration, etc.) PrtS is capable to hydrolyze αS1-casein in different sites of the fragment 1-23. However, with a pH 8 it presents cutting characteristics in those of PI, and with a pH 6 and with pH values close to those of cheese fermentation (5.2) and yogurt (4.0), the cuts are the same as those observed in PIII. This means that the cutting characteristics of this proteinase are a set of characteristics between PI and PIII of lactococci dependening on pH values [16, 31, 33]. This emphasizes that by changing the pH during the fermentation, the peptides toward PrtS can be modulated [34]. Besides these studies, the participation of PepS has been proven in the biosynthesis of peptidoglycan of the cell wall, which is essential for the development of *S. thermophilus* during fermentation [35].

Despite the previous information, not all the *S. thermophilus* strains possess PrtS, *e.g.*, strain CNRZ1066 lacks *prtS* gene and, in contrast, strain LMD-9 displays strong proteolytic activity due to the presence and expression of *prtS* gene [36]. It has been shown that for high proteolytic activity, the presence of a putative CodY-box is necessary in their promoter region, which is a feature of the transcriptional pattern of CodY-regulated genes that are able to bind promoters of 14 genes belonging to the proteolytic system, including PrtS [37]. It has been shown that the interaction between *S. thermophilus,* with or without PrtS, and Lactobacillus, especially *Lb. bulgaricus* in yoghurt production, leads to strong proteolytic activities. This is because *S. thermophilus* efficiently consumes the peptides produced by PrtB and remains the dominant population during fermentation [38]. Not always is PrtS action desirable, such as in the case of biofilm formation, while heating sections of cheese-milk pasteurization equipment, it creates an acidic flavor and undesirable texture. It has also been proved that with the absence of this enzyme no biofilm is formed [39].

Amino Acid and Peptide Transport System

The transport of amino acids and peptides, through the cell membrane, is the second stage in the proteolytic system of LAB. This step includes a great number of subsystems for certain amino acids [1, 3, 5, 18]. In the case of lactococci and lactobacilli, at least 10 different amino acid transport systems with a high specificity for amino acids with similar structures have been found, for example: Glu/Gln, Leu/Ile/Val, Ser/Thr, Ala/Gly, and Lys/Arg [3]. These systems can be regulated by ATP hydrolysis in the cases of Glu/Gln, Asn, and Pro/Gly [18], or by protons in the case of Leu/Val/Ile, Ala/Gly, Ser/Thr, and Met, while the Arg/Orn is regulated by a concentration gradient in a passive transport [1].

The transport system of *S. thermophilus* has been little studied. However, the complex requirements of amino acids dictate that growth of *S. thermophilus* depends on a highly efficient transport system [10]. This system works with an external source of energy because *S. thermophilus* lacks an endogenous reserve of energy to perform this transport [40]. In particular, two energy inputs for amino acid transport have been reported in this genus. The first one, ATP and ATP metabolites are used directly as energy sources; the second one finds its explanation in Mitchell's Chemiosmotic Theory, where the formation of a proton is needed to generate the motive force that will accompany the transport of amino acids and short-chain peptides. This amino acid transport system depends on temperature, and pH, where optimal temperature goes from 30°C to 45°C, and decreases rapidly over 50°C in *S. faecalis* [41].

Di- and Tri-Peptide Transport System

Di- and tri-peptide transport system is related to the transport of essential amino acids [42]. Studies on transport of hydrophilic di- and tripeptides in lactobacilli and lactococci have shown that this mechanism is regulated by the generation of protons. Dipeptide transport is carried out through an enzyme called DtpT that belongs to a family of enzymes called the PRT family. This group of enzymes is characterized by allowing the transport of hydrophilic di- and tri-peptides, which are unrelated to the peptide transporters in other bacteria belonging to the ABC family. Through the analysis of the genomic sequence encoding the DtpT enzyme, it is known that this group of enzymes requires ATP hydrolysis to perform its function [3].

Studies of the genome sequence of some strains of *S. thermophilus* have revealed a relationship between proton-dependent enzymes and some of the ATP-dependent enzymes, all of them included in the ABC family [10]. In *S. thermophilus*, most dipeptide-carrying enzymes are organized in a complex of five proteins, which is associated with the cell membrane [43].

A genomic DNA fragment of 5.8 kb of *S. thermophilus* (serotype M49 group A, strain CS101) was sequenced, and 5 genes coding for proteins of the Dpp system (*dppA* to *dppE*) were found. They had a similarity of 17% to 54% compared to the enzyme sequence of the ABC transport group [42]. The organization of the five genes encoding system was confirmed in *S. thermophilus*, where the most abundant was *dppA*, similar to that found in the oligopeptide permease system in lactococci [43]. To verify the role of each unit in the system of Dpp, Podbielski and Bettina [42] carried out an insertional inactivation to create two

Streptococcus species (M2 and M49), which did not express the genes *dppD* and *dppE*. They observed that in growth media with dipeptides, these species were unable to grow, hence their specificity towards dipeptides was detected.

Oligopeptide Transport System

The transport system of oligopeptides in LAB, particularly of lactobacilli and lactococci, has been one of the most studied [29]. It consists of five proteins (OppA, B, C, D, and F), belonging to the ABC family which are ATP-dependent, as in the case of the proteins from the transport systems of amino acids and peptides [13]. This system has not been fully characterized, but it is known for its ability to transport oligopeptides with up to 12 amino acids into the cell [44].

In contrast, the oligopeptide transport system of *S. thermophilus* (Ami) is considered a more complex system since it allows the entry of peptide chains of up to 23 amino acids [13]. In the Ami system, as well as in the Opp system, a larger number of proteins are involved. The Ami system of *Streptococcus thermophilus* has a high similarity with the Ami system of *S. pneumonia* [10]. The main difference between these systems is that the first one possesses two more oligopeptide-binding proteins. These binding proteins, which are ATP-dependent, are associated with the cell membrane and they are also responsible for the activation of operons (AmiA1, AmiA2 and AmiA3) [45].

The operon structure of the Ami system makes it more efficient than the Opp operon in terms of amino acid transport into the cell [46]. This higher efficiency compensates for the lack of a proteinase such as PrtP present in other LAB [19]. It has been shown that in the Ami system, long-chain peptides with a negative charge cannot be transported into the cell. On the other hand, peptide chains between 1,000 and 3,000 Da with a net positive charge and hydrophobic peptides can be transported more efficiently [13]. This system is complemented with two proteins bound to the cell wall (AmiC and AmiD). When the operon is activated, as occurs when sulfur amino acids are present in the medium, these proteins change to an α-helix conformation. This change allows them to act as a peptide binding permease, permitting the passing of peptides of up to 23 amino acids through the cell membrane. The energy required for their transport is also generated by the Ami operon through two ATP protein donors: AmiE and AmiF [43].

The existence of a gene regulatory system associated with the Ami system has been demonstrated. The gene regulatory mechanism involves the production, secretion

and transport of ComS and this peptide regulates the activity of the ComR protein, which is in turn a natural regulator of the *S. thermophilus* transcription system [47].

Peptidases of *S. thermophilus*

Once the peptides have been transported into the microorganism, they must be fractionated to release the essential amino acids. This action is performed through a complex system of intracellular peptidases and amino-peptidases. Several studies have been conducted to isolate and identify enzymes that participate in this system. Their sequences, specificity and physicochemical characteristics have been described [29]. The proteolytic system of *S. thermophilus* consists of fourteen peptidases, of which, PepN, PepC, PepS, PepX, and PepO, have different characteristics and specificity compared to those of other LAB [48].

Aminopeptidase N (PepN). This peptidase of *S. thermophilus* is a 95 kDa monomer with a maximum activity at pH 7 and 37°C. This peptidase is strongly inhibited by chelating agents, suggesting that it is a metallopeptidase. Furthermore, it has been shown that the presence of divalent ions such as Co^{2+}, Zn^{2+}, Mn^{2+} are able to restore its activity. This aminopeptidase has a high specificity for hydrophobic amino acids, particularly at the N-terminal, in low molecular weight peptides (chains of 4 to 6 amino acids). These characteristics placed it within the family of zinc metallopeptidase [49].

In contrast, aminopeptidase of *L. lactis* shows a preference for arginine dipeptide on the N-terminal residue but it is also capable of hydrolyzing peptides having lysine and leucine. This enzyme is able to cut amino acid N-terminal residue in a wide range of sizes and compositions. Its activity has been directed to the hydrolysis of oligopeptides with proline at P1 and P2 [50]. Transcription analyses have proved that this aminopeptidase shows high homology with other peptidases reported in LAB [51, 52]. It has been revealed that the sequences encoding for this enzyme are kept in almost all LAB [53–55].

Aminopeptidase C (PepC). *S. thermophilus* PepC is similar to the lactococcal peptidase. This aminopeptidase has specificity for peptides with cysteine residues. One of the most significant differences between these peptidases is that aminopeptidase of *S. thermophilus* is not able to release proline residues at pH 7 and 37°C [28].

Chapot-Chartier *et al*. [56] found that PepC of *S. thermophilus* has 70% homology with the sequence of PepC of *Lactococcus lactis* subsp. *cremoris*. Furthermore, it has been confirmed that the catalytic site of *S. thermophilus*

CNRZ 302 aminopeptidase maintains the same region for the cysteine proteinase of *Lactococcus lactis* subsp. *cremoris*. The biochemical characterization of this enzyme confirmed that the cloned peptidase is a thiol aminopeptidase with a molecular weight of 300 kDa with a hexameric structure.

Aminopeptidase C is usually a thiol peptidase of about 50 kDa in all the LAB [54], which hydrolyzes dipeptides with Ala, Leu or Lys in the N-terminal position while being incapable of hydrolyzing hexapeptides. A notable difference in terms of the quaternary structure is the organization of the hexamer shown by the PepC of *Streptococcus thermophilus*, compared to tetramers organization in other species of LAB [3].

Peptidase PepS. This is a monomeric metallopeptidase of 45 kDa which acts at pH between 7.5 and 8.5, and at a temperature of 55°C. It is characterized by being highly specific for peptides with arginine or aromatic amino acids at the N-terminal of the polypeptidic chain [19]. This aminopeptidase has a high homology with the family of intracellular aminopeptidases T of thermophilic bacteria (40% to 50%), therefore it is classified within this family [57]. There are several examples of evidence where the molecular mass (45 kDa) of the gene coding for this peptidase is very similar to that of other enzymes purified from the same family. Likewise, studies of the gene coding have shown there is no difference in the N- terminal sequence of PepS and other thermophilic enzymes [58]. Based on the amino acid sequence of PepS, it is assumed that it is intracellular [59].

Additionally, there is no associated hydrophobic sequence as it is found in the extracellular enzymes [60]. PepS can participate in the hydrolysis of peptides with high concentration of hydrophobic amino acids and thus releases smaller peptides with aromatic amino acids [61–63].

Peptidase PepX. This peptidase was purified first by Tsakalidou *et al.* [64] from *Streptococcus thermophilus* ACA-DC4. PepX is a peptidase that is classified in the same group of serine proteases and has been called aminopeptidase X-prolyl-dipeptidyl [65]. The specificity of this peptidase is over proline, and regardless of the adjacent amino acid, it hydrolyses the peptide bond at carboxyl side of the proline residue, cleaving dipeptidyl residue when this amino acid is the penultimate N-terminal residue [4, 66]. This enzyme consists of two subunits, each one with a molecular mass of 80 kDa. Its optimum activity is at pH 7 and 50°C and it can be inactivated by compounds with sulfhydryl groups [66].

PepX isolated from LAB and other bacteria, yeast, and eukaryotes has been classified as serine proteinase based on the inhibitory effect of several compounds [64, 67–70].

The activation or inactivation of peptidases has been studied because it activates cheese ripening and yoghurt fermentation. Giannoglou *et al.* [71] showed that emergent technologies, such as high pressure, have the ability to increase or inactivate PepX depending on the range of pressure and temperature used, making small or high changes in the enzyme structure.

Oligopeptidase PepO. Oligopeptidase PepO of *S. thermophilus* is a metallopeptidase of 70 kDa, with a maximum activity at pH 6.5 and 41°C. This peptidase acts on oligopeptide chains of 5 to 30 amino acids and its specificity makes PepO a unique peptidase in the proteolytic system of *S. thermophilus* although this enzyme has certain homology with peptidases of the same group of other LAB [72], like that of *L. lactis* PepN proteolytic activity measured in vitro [51]. PepO activity is specific for peptides with arginine and methionine in the polypeptide chain [72, 73].

The proteolytic system of *S. thermophilus* is involved in the release of peptidic sequences from caseins and whey proteins during lactic acid fermentation. This microorganism is associated with other lactic acid bacteria worldwide in the production of fermented dairy products. Additionally, the whole action of each proteolytic system results in the release of amino acids that supply the metabolic needs of the microorganisms and potentially bioactive peptides. The study of bioactive peptides has developed much in the last decade; hence, the importance of knowing more about the characteristics of the proteolytic system of *S. thermophilus*.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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