

Effect of growth phase and growth medium on peptidase activities of starter lactic acid bacteria

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Abstract – Selected, highly proteolytic strains (*Lactobacillus casei* ssp. *casei* RP0, *Lactobacillus helveticus* M10, *Lactobacillus delbrueckii* ssp. *bulgaricus* 2-11 and *Streptococcus thermophilus* TEP4) isolated from prime Kashkaval cheese, produced from raw milk in different regions of the Rhodopi Mountains in Bulgaria, were evaluated for peptidase activities (amino-, di-, tri- and endopeptidase) with 19 substrates. The effect of the growth phase and growth medium on the peptidase activities of the cell-free extracts of the strains, prepared from the mid-log phase, late-log phase and stationary phase of the cells grown in 12% reconstituted skim milk (RSM) or MRS/M17 broth were investigated. *L. casei* ssp. *casei* RP0 and *L. helveticus* M10 possessed by far the highest hydrolase activity, which was an indication of the presence of powerful aminopeptidases, X-prolyl-dipeptidyl aminopeptidases, proline iminopeptidases and tripeptidases. *L. delbrueckii* ssp. *bulgaricus* 2-11 had high hydrolase activity toward proline-containing substrates (alanyl-prolyl- ρ -nitroanilide and prolyl- ρ -nitroanilide). The most distinct difference between the peptidase activities of the lactobacilli and *S. thermophilus* TEP4 was exhibited toward the Glu-Glu substrate. The latter was very effectively hydrolyzed by *S. thermophilus* TEP4. The specific activities of most peptidases (over 90%) were significantly influenced by the growth phases and growth medium. The highest levels of peptidase activity of all lactic acid bacteria were obtained with cells grown in RSM in the late-log phase.

growth phase / growth medium / peptidase activities / lactic acid bacteria / starters

摘要 – 生长期和培养基对乳酸菌发酵剂产肽酶活性的影响。从保加利亚 Rhodopi 山地区鲜奶生产的 Kashkaval 干酪中分离和筛选出蛋白水解活性高的菌株 *Lactobacillus casei* ssp. *casei* RP0、*Lactobacillus helveticus* M10、*Lactobacillus delbrueckii* ssp. *bulgaricus* 2-11 和 *Streptococcus thermophilus* TEP4, 并用 19 种诊断物质评价了这些菌株产肽酶(氨基-, 二-, 三-和肽链内切酶)的活性。在 12% 的还原脱脂乳或 MRS/M17 培养基中, 研究了培养期(对数增长中期和后期) 和培养基对胞内肽酶活力的影响。*L. casei* ssp. *casei* RP0 和 *L. helveticus* M10 菌株产水解酶的活力最高, 在水解酶中存在大量的氨基肽酶、X-脯氨酰-二肽氨基肽酶、脯氨酸-亚氨基肽酶和三肽酶。*L. delbrueckii* ssp. *bulgaricus* 2-11 在含脯氨酸底物(丙氨酰基-脯氨酰- ρ -硝基苯胺和-脯氨酰- ρ -硝基苯胺)中具有较高的水解酶活性。在谷氨酸-谷氨酸底物中乳杆菌和嗜热链球菌 TEP4 的肽酶活性表现出非常明显的不同。谷氨酸-谷氨酸底物可以被 *S. thermophilus* TEP4 非常有效地水解。多数肽酶(超过 90%) 的比活力显著地受生长期和培养基的影响。所有乳酸菌在 12% 还原脱脂乳粉培养基及对数生长后期时获得的胞内肽酶活力最高。

生长期 / 培养基 / 肽酶活性 / 乳酸菌 / 发酵剂

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Résumé – Effet de la phase de croissance et du milieu de culture sur les activités peptidasiques de bactéries lactiques. Des souches sélectionnées pour leur activité protéolytique élevée (*Lactobacillus casei* ssp. *casei* RP0, *Lactobacillus helveticus* M10, *Lactobacillus delbrueckii* ssp. *bulgaricus* 2-11, *Streptococcus thermophilus* TEP4) isolées de fromage Kashkaval de bonne qualité, produit à partir de lait cru dans différentes régions des montagnes de Rhodopi en Bulgarie, ont été évaluées pour leur activités peptidasiques (amino-, di-, tri- et endopeptidase) sur 19 substrats. L'effet de la phase de croissance et du milieu de culture sur les activités peptidasiques des extraits intracellulaires des souches a été étudié en milieu et en fin de phase exponentielle, et en phase stationnaire de culture sur lait écrémé reconstitué à 12 % (RSM) ou sur milieu de culture MRS/M17. *L. casei* ssp. *casei* RP0 et *L. helveticus* M10 possédaient de loin l'activité hydrolytique la plus élevée, indiquant la présence d'aminopeptidases très actives, d'X-prolyl-dipeptidyl aminopeptidases, de proline imino-peptidases et de tripeptidases. *L. delbrueckii* ssp. *bulgaricus* 2-11 avait une activité hydrolytique élevée sur les substrats synthétiques contenant de la proline (alanyl-prolyl- ρ -nitroanilide et prolyl- ρ -nitroanilide). La différence la plus nette entre les activités peptidasiques des lactobacilles et de *S. thermophilus* TEP4 était observée sur le substrat Glu-Glu, ce dernier étant bien hydrolysé par *S. thermophilus* TEP4. Les activités spécifiques de la plupart des peptidases (plus de 90 %) étaient significativement influencées par les phases de croissance et le milieu de culture. Les niveaux les plus élevés d'activité peptidasique de toutes les bactéries lactiques étaient obtenus avec des cellules cultivées sur RSM et à la fin de la phase exponentielle de croissance.

phase de croissance / milieu de croissance / activité peptidasique / bactérie lactique / levain

1. INTRODUCTION

The proteolytic system of lactic acid bacteria is crucial for their growth in milk [3, 5, 40, 42] and for texture and flavor formation in cheeses [9, 20, 29, 41, 44]. It is a multi-enzyme system comprising membrane-connected proteinase, peptidases and transport systems [26, 37, 39]. The most extensively researched is the proteolytic system of lactococci (*Lc. lactis* and *Lc. cremoris*) [25, 49]. Recent studies have irrefutably shown the intracellular localization of all *Lactococcus* peptidases [33, 50]. Extracellular hydrolysis of casein is believed to be carried out only by PrtP proteinase, which produces sufficient di-, tri- and oligopeptides directly assimilated by the cells to support growth of bacteria in milk [25, 36]. In contrast to lactococci, the proteolytic system of lactobacilli has not been thoroughly investigated [6, 21, 23, 28, 42]. In recent years, researchers have recognized the importance of the proteolytic system of lactobacilli for cheese ripening and cheese quality [9, 10, 18, 22]. Despite the intracellular location of *Lactobacillus* peptidases, the authors accentuate their crucial role in

flavor development in cheeses [54, 55] in relation to cell lysis releasing all cytoplasmic enzymes [1, 25, 30]. It has been shown that the *L. delbrueckii* ssp. *bulgaricus* and *L. helveticus* strains undergo lysis during ripening of Cheddar cheese and release their intracellular enzymes, which, in turn, enhance proteolysis and improve cheese flavor [22]. A series of studies on the proteolytic activity of *L. bulgaricus* and *L. helveticus* strains have proved their favorable effect on the ripening of different cheeses [20, 38, 41, 46]. The exceptional characteristics of the dynamics of amino acid release by some *Lactobacillus* strains could be due to an effective proteolytic system [6, 19, 21]. The inclusion of peptidolytic strains in starter cultures or using them as adjunct (nonstarter) cultures for cheeses indicates the release of free amino acids during ripening, which not only contribute directly to cheese flavor formation, but also act as precursors of other important flavor and aroma components [29, 43, 55]. They manipulate the overall cheese flavor profile, and the rate of flavor formation [13, 46].

There has been a growing interest in the proteolytic systems of lactobacilli

and streptococci owing to their wider inclusion as starter cultures in fermented milks [21,45,48]. It has been reported that the increase in the proteolytic status of the starter cultures heightens the final level of cell concentration, and the concentration of free amino acids in yogurt and kefir [2, 47]. In mixed culture the proteolytic activity of *L. bulgaricus* is higher than that of *S. thermophilus* [48], and it has been proved that the free amino acids produced by *L. bulgaricus* are utilized by *S. thermophilus* [2,48], and that there is a growth connection between them [48]. Casein metabolism in yogurt culture results mainly from the endopeptidase activity of *L. bulgaricus* [48]. Although *S. thermophilus* is widely used in the dairy industry, its proteolytic system is not well characterized. *S. thermophilus* strains are described as slightly proteolytic, manifesting a strong necessity of amino acid nitrogen in milk for growth and metabolism [14,16,27]. *S. thermophilus* exhibits a very low membrane-bound proteinase activity; its proteolytic system mainly consists of peptidases [23,40,45].

Biosynthesis of *Lactobacillus* and *Lactococcus* proteinases and peptidases depends on the culture medium, species, and on the strain itself [19, 31, 35]. Peptidase activities of strains of lactic acid bacteria grown mainly in broth media were studied in various growth phases [22, 45, 54, 55]. Comprehensive studies on the effect of the growth phase and growth medium on the level of peptidase activities of industrial starter strains of lactic acid bacteria have not been done.

The purpose of the present study was to evaluate the peptidolytic potential of four strains of lactic acid bacteria (*L. delbrueckii* ssp. *bulgaricus*, *L. casei* ssp. *casei*, *L. helveticus* and *S. thermophilus*), included in starter cultures for manufacturing Bulgarian dairy products, as a primary criterion in selecting strains for the dairy industry; to evaluate the impact of the growth

phase and growth medium on peptidase activity.

2. MATERIALS AND METHODS

2.1. Bacterial strains

The strains *L. delbrueckii* ssp. *bulgaricus* 2-11, *L. helveticus* M10, *L. casei* ssp. *casei* RP0 and *S. thermophilus* TEP4 of the culture collection of the Laboratory of Applied Microbiology at the Institute of Microbiology (Bulgarian Academy of Sciences) were used in the present study. They were selected by means of a screening process based on the proteolytic activity of 921 strains of lactic acid bacteria isolated from well-matured batches of Kashkaval cheese and white-brined cheese produced from raw milk in ecological regions of the Rhodopi Mountains, and from home-made Bulgarian yogurt. The species identification of the four strains of lactic acid bacteria was performed on the basis of nucleotide sequencing of the 16S ribosomal gene – DNA-fragment characteristic for 4 strains obtained after PCR with species-specific primers. Each culture of strains was maintained in 12% sterile reconstituted skim milk (RSM) supplemented with 2% glucose (Fluka RdH, Buchs, Switzerland) and 1% yeast extract (Fluka RdH, Buchs, Switzerland) and stored at –80 °C. Working cultures were prepared from frozen cultures by three successive transfers in 12% (w/v) low-heat RSM before use, and stored at 4 °C.

2.2. Proteolytic activity in reconstituted skim milk

All the bacterial strains were grown overnight at 37 °C (*L. bulgaricus* 2-11, *L. helveticus* M10 and *S. thermophilus* TEP4) and at 30 °C (*L. casei* RP0) in MRS/M17 broth (Merck, Darmstadt, Germany). To minimize carryover of free

amino acids during inoculation, 5 mL of cells were washed and resuspended to the original volume with 0.32 mmol·L⁻¹ sodium phosphate, pH 7.2. Cells were inoculated (2%) into RSM 12% (w/v) and incubated at their optimum temperatures (37 °C for *L. bulgaricus* 2-11, *L. helveticus* M10 and *S. thermophilus* TEP4, and 30 °C for *L. casei* RP0) for 6 h. A control consisted of uninoculated RSM. A 2.5-mL sample of each incubated RSM was mixed with 10 mL of 0.75 mol·L⁻¹ trichloroacetic acid (TCA) and 1 mL of water to 5 mL of sample to give a final concentration of 0.47 mol·L⁻¹ (7.7%) TCA. The samples were filtered using a Whatman number 4A filter paper after 10 min of incubation at room temperature (~22 °C) and frozen at -80 °C until assayed. The *o*-phthaldialdehyde (OPA) method described by Church et al. [7] was used to determine the concentration of free amino groups in the filtrate. Triplicate aliquots from each TCA filtrate were analyzed using a spectrophotometer (Shimadzu AA-6200, Shimadzu Corporation, Tokyo, Japan). The proteolytic activity of these bacterial cultures is expressed as the amount of free α -amino groups measured as difference in absorbance values at 340 nm after subtraction of values for the uninoculated control RSM.

2.3. Harvesting of microbial cells and preparation of cell-free extracts

The bacterial strains were grown in RSM and MRS broth (pH 5.4) for the lactobacilli at their optimum temperatures (37 °C for *L. bulgaricus* 2-11 and *L. helveticus* M10, 30 °C for *L. casei* RP0) and in RSM and M17 broth (pH 6.3) for *S. thermophilus* TEP4 (37 °C) with 1% inoculum for each culture up to the desired growth phases.

Once the desired growth phase was reached (mid-log phase for MRS broth/M17 broth at pH 4.6 and at pH 5.2 for RSM; late-log phase for MRS broth/M17 broth at pH 4.2 and at pH 4.7 for RSM; stationary phase after 22 h, 28 h and 36 h of incubation in MRS broth for *L. bulgaricus* 2-11, *L. helveticus* M10 and *L. casei* RP0, 24 h incubation in M17 broth for *S. thermophilus* TEP4, and after 7, 14, 22 and 6 h of incubation for *L. bulgaricus* 2-11, *L. helveticus* M10, *L. casei* RP0 and *S. thermophilus* TEP4, respectively, in RSM) the cells were collected from the growth medium by centrifugation at 12 000× *g* for 15 min at 4 °C. The pellet of each strain was triple-washed with 40 mL of 50 mmol·L⁻¹ Tris-HCl (pH 7.5). The washed cells were then resuspended in 10 mL of 50 mmol·L⁻¹ Tris-HCl (pH 7.5) and sonicated (Branson Sonifier 250, Danbury, CT, USA) at 30-s intervals for 5 min at 4 °C. Cell debris was removed by centrifugation at 12 000× *g* for 15 min at 4 °C. The supernatant was used for enzyme assays.

The protein concentrations of the samples were determined by the Bradford staining method [4]. Calibration was carried out using bovine serum albumin (Fluka RdH, Buchs, Switzerland).

2.4. Enzyme assays

2.4.1. Aminopeptidase activity

The aminopeptidase activities were assayed with chromogenic substrates: amino acid derivatives of ρ -nitroanilide (ρ Na) – Lys- ρ Na, Leu- ρ Na, Met- ρ Na, Ala- ρ Na, Arg- ρ Na, Glu- ρ Na (PepA, aminopeptidase A), Pro- ρ Na (PepI, proline iminopeptidase) and Ala-Pro- ρ Na (PepX, X-prolyl-dipeptidyl aminopeptidase) using the method of Fernandez-Esplá et al. [12]. Aminopeptidase activity was assayed by incubation of 100 μ L of sample

with 400 μL of 50 $\text{mmol}\cdot\text{L}^{-1}$ Tris-HCl buffer, pH 7.0 and 50 μL of 10 $\text{mmol}\cdot\text{L}^{-1}$ of substrate at 37 °C (*L. helveticus* M10, *L. bulgaricus* 2-11 and *S. thermophilus* TEP4) or 30 °C (*L. casei* RP0) for 20 min. The reaction was stopped by addition of 1 mL of 30% acetic acid. Activities were quantified by the concentration of the relevant ρ -nitroanilide to absorbance at 410 nm under the assay conditions. The concentration of ρ -nitroanilide was calculated by using a molar absorbance coefficient of 9024 $\text{mol}^{-1}\cdot\text{cm}^{-1}$. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of ρ -nitroanilide per min under the above conditions of the assay. The specific activity was expressed as $\mu\text{moles } \rho\text{-nitroanilide released}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$.

2.4.2. Dipeptidase activity

Dipeptidase activities were quantified using Leu-Gly, Ala-His, Leu-Tyr, Glu-Glu (PepA, glutamyl aminopeptidase), Leu-Pro (PepQ, prolidase) and Pro-Leu (PepR, prolinase) as substrates according to the method of Wohlrab and Bockelmann [56]. The reaction mixture contained 10 μL of enzyme solution + 415 μL of 50 $\text{mmol}\cdot\text{L}^{-1}$ Tris-HCl buffer, pH 7.5 + 50 μL of 22 $\text{mmol}\cdot\text{L}^{-1}$ substrate + 25 μL of peroxidase (5 $\text{mg}\cdot\text{mL}^{-1}$ in 0.8 $\text{mol}\cdot\text{L}^{-1}$ $(\text{NH}_4)_2\text{SO}_4$) + 25 μL of L-amino acid oxidase (2 $\text{mg}\cdot\text{mL}^{-1}$ in distilled water) + 25 μL of *o*-dianisidine (11.5 $\text{mmol}\cdot\text{L}^{-1}$). The test tubes containing reaction mixture were incubated at 50 °C for 20 min. The reactions were stopped by the addition of 50 μL of dithiothreitol (120 $\text{mmol}\cdot\text{L}^{-1}$). Oxidation of *o*-dianisidine coupled to substrate hydrolysis resulted in an increase in brown color which was measured at 436 nm. Enzyme activity was calculated using a molar absorbance coefficient of 8100 $\text{mol}^{-1}\cdot\text{cm}^{-1}$ for oxidized

o-dianisidine [52]. The specific activity was expressed as $\mu\text{moles amino acid released}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$.

2.4.3. Tripeptidase and various proline-specific peptidase activities

Certain peptidase activities were quantified using the modified colorimetric ninhydrin method of Doi et al. [8] in which free amino acids are determined by hydrolysis of the non-chromogenic synthetic peptides: Leu-Leu-Leu (PepT, tripeptidase), Arg-Pro-Pro (PepP, aminopeptidase), Pro-Leu-Gly-Gly (PepI) and Pro-Gly-Gly (PepI, tripeptidase). Prior to carrying out these assays, CFE_s (2.5 mL) were passed through a Sephadex PD 10 gel permeation column (Pharmacia Biotech, Uppsala, Sweden) with Sephadex G-25M in order to remove amino acid residues, and thus avoid high blank values with the cadmium-ninhydrin reagent. The Sephadex column was equilibrated with 50 $\text{mmol}\cdot\text{L}^{-1}$ Tris-HCl (pH 7.5) and the sample was eluted with 3.5 mL 50 $\text{mmol}\cdot\text{L}^{-1}$ Tris-HCl containing 100 $\text{mmol}\cdot\text{L}^{-1}$ NaCl. After 1 h of incubation of the CFEs with substrates at 30 °C, the reaction was stopped by the addition of 0.65 mL of 24% TCA. A sample of TCA supernatants (20–100 μL , depending on the concentration of amino acids) was added to 1.7 mL Cd-ninhydrin reagent (0.8 g ninhydrin were dissolved in a mixture of 80 mL 99.5% ethanol and 10 mL acetic acid, followed by the addition of 1 g CdCl_2 dissolved in 1 mL of distilled water). The mixture was heated at 84 °C for 5 min, then cooled and the absorbance at 506 nm determined. Activities were quantified by reference to a standard curve relating the concentration of the relevant amino acid, calculated using the molar absorption coefficient of leucine. Activity hydrolyzing Leu-Leu-Leu was terminated after 15 min of incubation. Activities

hydrolyzing Pro-Leu-Gly-Gly (PepI) were quantified using a standard curve generated with proline, and absorbance was read at 440 nm. The specific peptidase activity was expressed as nmoles amino acid released·mg protein⁻¹·min⁻¹.

2.4.4. Oligopeptidase F (PepF)

PepF activity was determined with bradykinin using the method of Monnet et al. [34]. The activity was routinely measured by incubating the enzyme solution with bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg; 0.23 mmol·L⁻¹ final concentration) in 50 mmol·L⁻¹ Tri-HCl buffer pH 8 at 40 °C. The digestion was stopped by addition of trifluoroacetic acid to 1% final concentration. The reaction was followed by reverse-phase high-performance liquid chromatography (RP-HPLC) using a C18 Vydac column (250 mm × 4.6 mm, Touzart et Matignon, Vitry-sur-Seine, France) in a trifluoroacetic acid/acetonitrile solvent system (solvent A = 0.115% trifluoroacetic acid; solvent B = 0.1% trifluoroacetic acid, CH₃CN 60%) with a linear gradient from 20 to 80% solvent B in 15 min. The absorbance at 214 nm allowed the measurement of peak areas which were then converted into amounts of peptide using Arg-Pro-Pro-Gly-Phe-Pro as a standard. One unit of endopeptidase activity was defined as the amount of enzyme releasing 1 μmol of the peptide Arg-Pro-Pro-Gly-Phe per min. The specific activity was expressed as μmoles peptide released·mg protein⁻¹·min⁻¹.

2.5. Statistical analysis

During quantification of peptidase activities triplicate analysis of independent triplicate CFEs was carried out, and results were reported as means ± standard error. Statistical analysis was undertaken using the software SigmaPlot 2001.

3. RESULTS

3.1. Growth phase effect on the peptidase activities of *L. bulgaricus* 2-11, *L. helveticus* M10, *L. casei* RP0 and *S. thermophilus* TEP4

From 921 strains of lactic acid bacteria isolated by means of a screening process based on the total proteolytic activity, 38 strains were selected. The strains were genetically identified and classified. 16 of the selected strains belonging to the species *L. delbrueckii* ssp. *bulgaricus*, *L. helveticus*, *L. casei* ssp. *casei* and *S. thermophilus* were associated with starter cultures for traditional Bulgarian dairy products – Bulgarian yogurt, white-brined cheese and Kashkaval cheese; 8 strains were used as nonstarter (adjunct) cultures in Kashkaval manufacture. From the selected strains, 3 *Lactobacillus* strains (*L. bulgaricus* 2-11, *L. casei* RP0 and *L. helveticus* M10) and 1 streptococcal strain (*S. thermophilus* TEP4), possessing the highest proteolytic activity, were chosen and included in starter cultures for yogurt manufacture (*L. bulgaricus* 2-11 and *S. thermophilus* TEP4), for Kashkaval cheese (*L. casei* RP0, *L. helveticus* M10 and *L. bulgaricus* 2-11) and for white-brined cheese (*L. casei* RP0) to study their peptidolytic profiles.

Figure 1 represents the proteolytic activities in RSM of 3 *Lactobacillus* strains (*L. casei* RP0, *L. helveticus* M10 and *L. bulgaricus* 2-11) and 1 *S. thermophilus* TEP4 strain, which manifested the highest activity of α-amino group release as a result of the proteolysis of milk proteins from 38 selected lactic acid bacteria. The proteolytic activity of *Lactobacillus* was significantly higher than that of *S. thermophilus* TEP4. Among the selected *S. thermophilus* strains, *S. thermophilus* TEP4 was highly proteolytic, producing the highest amount of free

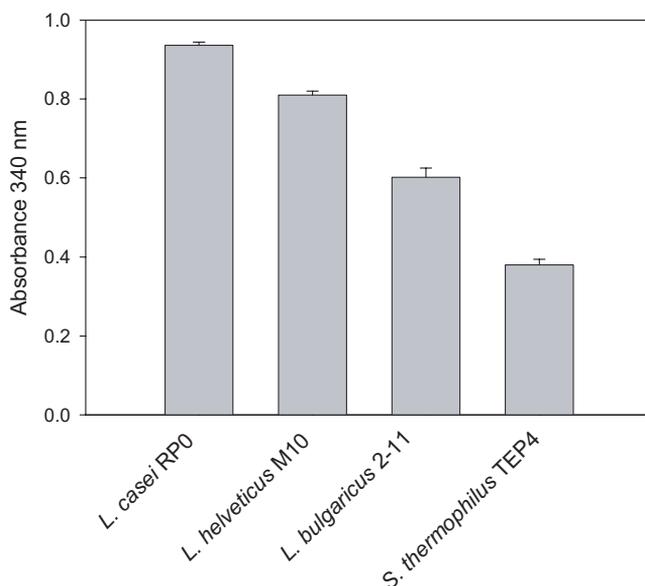


Figure 1. Indication of proteolytic activity of *L. casei* RP0, *L. helveticus* M10, *L. bulgaricus* 2-11 and *S. thermophilus* TEP4 strains after incubation in RSM at 30 °C and 37 °C for 6 h. Data represent differences in absorbance values after subtracting the value for the uninoculated control RSM.

amino groups ($77.8 \mu\text{mol}\cdot\text{L}^{-1}$). Among the lactobacilli, *L. casei* RP0 showed the highest proteolytic activity, releasing the highest amount of free amino groups ($208.8 \mu\text{mol}\cdot\text{L}^{-1}$), followed by *L. helveticus* M10 ($187.9 \mu\text{mol}\cdot\text{L}^{-1}$). *L. bulgaricus* 2-11 possessed the lowest producing activity of free amino groups among the lactobacilli ($143.6 \mu\text{mol}\cdot\text{L}^{-1}$).

Figures 2, 3, 4, 5 and 6 show the specific activities of different hydrolase activities in cell-free extracts (CFEs) of the given strains, whose cells were harvested at the mid-log phase, late-log phase and stationary phase of cell growth in reconstituted skim milk (RSM) (Histograms A) and in MRS/M17 broth (Histograms B). 19 activities were significantly ($P < 0.05$) influenced by the growth phase in both media. Five aminopeptidase activities, 3 dipeptidase, 1 tripeptidase, 7 proline-specific activities and 1 oligopeptidase activity of *L. bulgaricus* 2-11 were

significantly ($P < 0.05$) influenced by the growth phase during cultivation in skim milk and in MRS broth (Fig. 2). The levels of peptidase activities of the strain in the late-log phase were significantly ($P < 0.05$) higher than the activities in the mid-log phase and stationary phase of growth in milk and in MRS broth (except aminopeptidase activities, hydrolyzing Lys- ρ Na and Leu- ρ Na (Fig. 2B), and tripeptidase activity, hydrolyzing Leu-Leu-Leu (Fig. 2B)).

The strains *L. helveticus* M10 and *L. casei* RP0 possessed exceptionally high hydrolase activity, exceeding that of *L. delbrueckii* ssp. *bulgaricus* 2-11 by 1.5–9.0 times in the late-log phase. The peptidase activities of *L. casei* RP0, hydrolyzing Lys- ρ Na, Leu- ρ Na, Ala-Pro- ρ Na, Leu-Pro and Leu-Leu-Leu ($1781.73 \pm 20.67 \mu\text{mol}\cdot\text{mg protein}^{-1}$, $910.11 \pm 20.40 \mu\text{mol}\cdot\text{mg protein}^{-1}$, $810.33 \pm 18.6 \mu\text{mol}\cdot\text{mg protein}^{-1}$, $998.86 \pm 4.49 \mu\text{mol}\cdot\text{mg protein}$ and $1663.12 \pm$

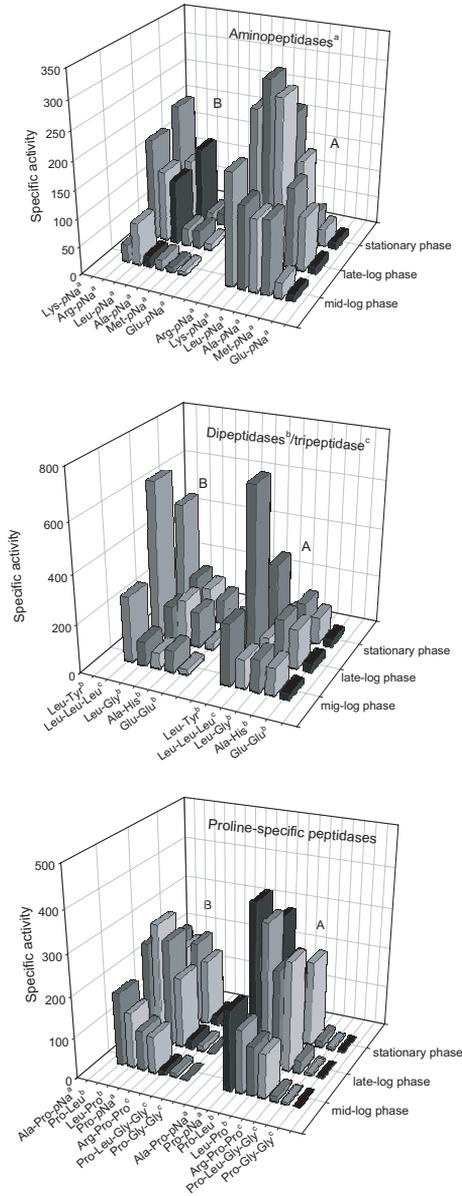


Figure 2. Peptidase activities in cell-free extracts of *L. bulgaricus* 2-11 from mid-log-phase, late-log-phase and stationary-phase cells grown in either RSM (Histograms A) or MRS broth (Histograms B). Values are the means of triplicate analysis of CFEs prepared in independent triplicate. ^aSpecific activity expressed as $\mu\text{moles } \rho\text{Na released}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$. ^bSpecific activity expressed as $\mu\text{moles amino acid released}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$. ^cSpecific activity expressed as $\text{nmoles amino acid released}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$.

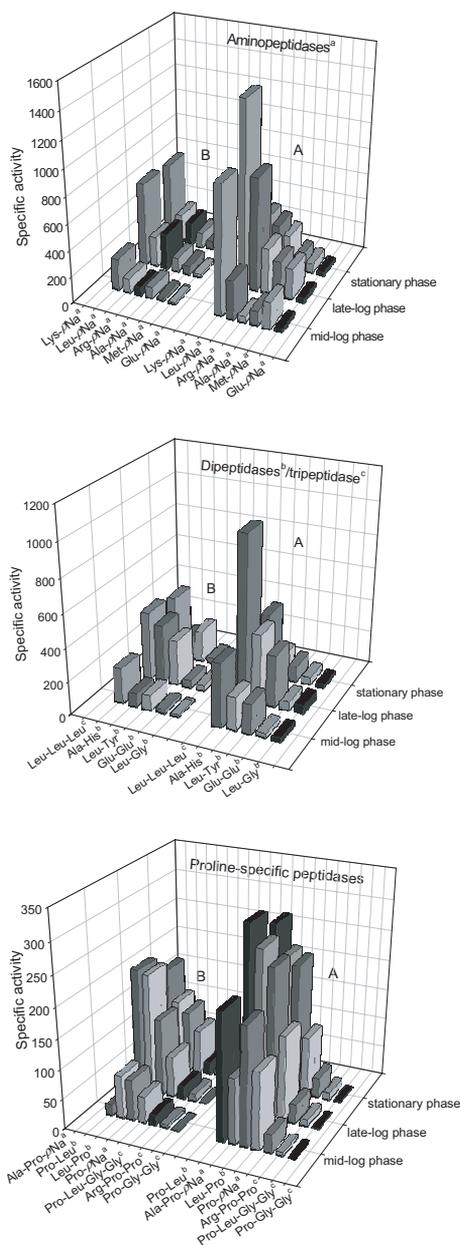


Figure 3. Peptidase activities in cell-free extracts of *L. helveticus* M10 from mid-log-phase, late-log-phase and stationary-phase cells grown in either RSM (Histograms A) or MRS broth (Histograms B). Values are the means of triplicate analysis of CFEs prepared in independent triplicate. ^aSpecific activity expressed as $\mu\text{moles } \rho\text{Na released}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$. ^bSpecific activity expressed as $\mu\text{moles amino acid released}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$. ^cSpecific activity expressed as $\text{nmoles amino acid released}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$.

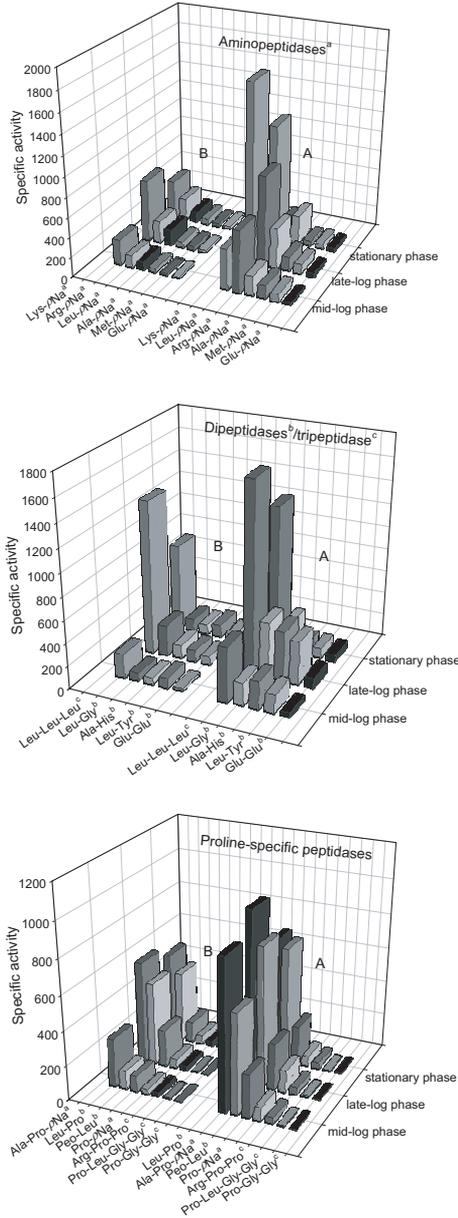


Figure 4. Peptidase activities in cell-free extracts of *L. casei* RPO from mid-log-phase, late-log-phase and stationary-phase cells grown in either RSM (Histograms A) or MRS broth (Histograms B). Values are the means of triplicate analysis of CFEs prepared in independent triplicate. ^aSpecific activity expressed as $\mu\text{moles } \rho\text{Na released}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$. ^bSpecific activity expressed as $\mu\text{moles amino acid released}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$. ^cSpecific activity expressed as $\text{nmoles amino acid released}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$.

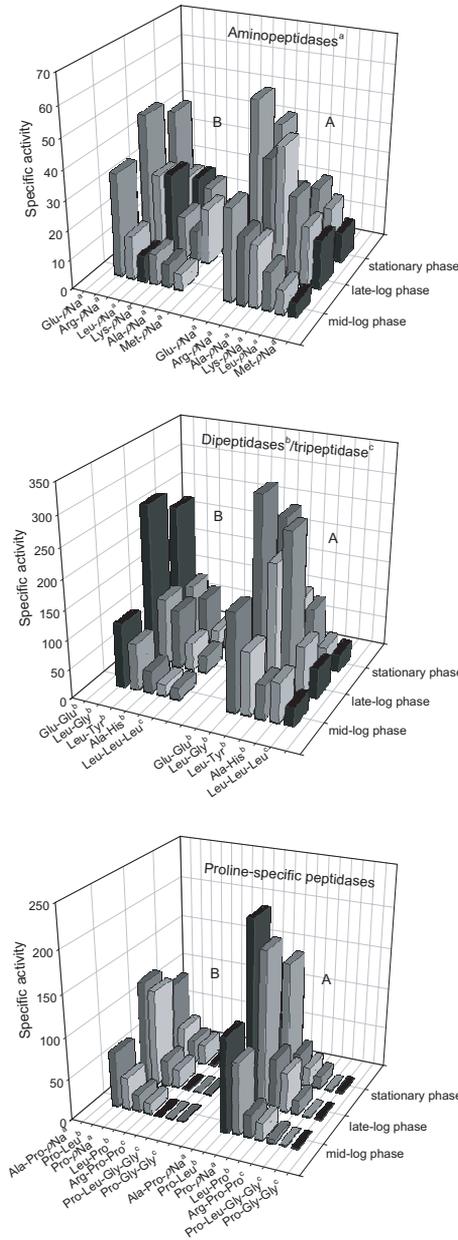


Figure 5. Peptidase activities in cell-free extracts of *S. thermophilus* TEP4 from mid-log-phase, late-log-phase and stationary-phase cells grown in either RSM (Histograms A) or M17 broth (Histograms B). Values are the means of triplicate analysis of CFEs prepared in independent triplicate. ^aSpecific activity expressed as $\mu\text{moles } \rho\text{Na released} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$. ^bSpecific activity expressed as $\mu\text{moles amino acid released} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$. ^cSpecific activity expressed as $\text{nmoles amino acid released} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$.

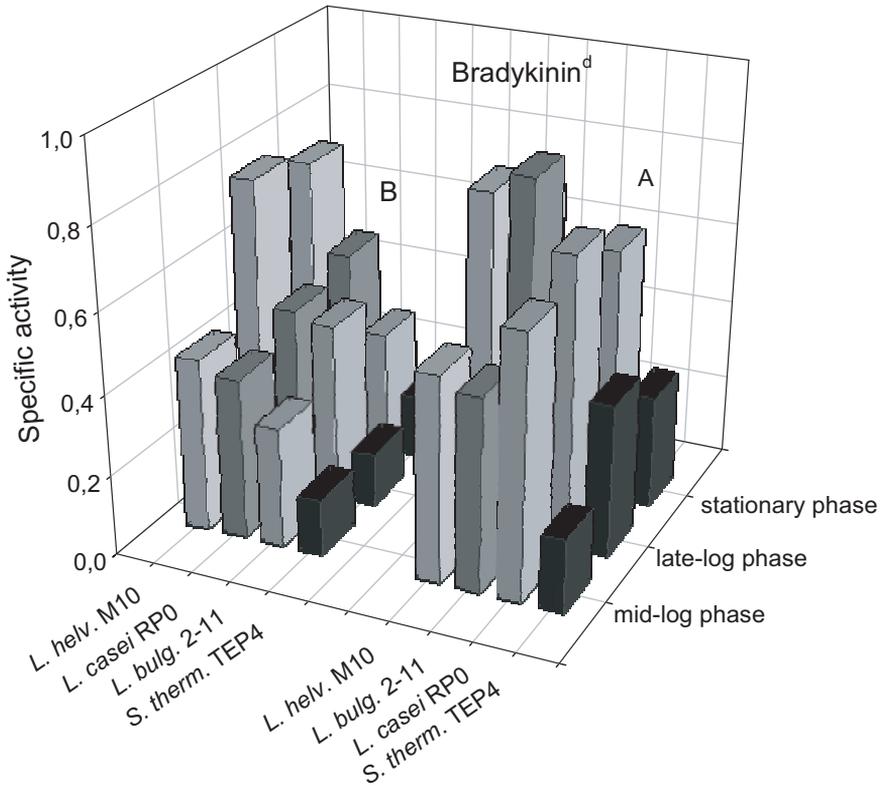


Figure 6. Oligopeptidase activities in cell-free extracts of LAB from mid-log-phase, late-log-phase and stationary-phase cells grown in either RSM (Histograms A) or MRS/M17 broth (Histograms B). Values are the means of triplicate analysis of CFEs prepared in independent triplicate. ^dSpecific activity expressed as $\mu\text{moles peptide released}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$.

2.70 $\text{nmol}\cdot\text{mg protein}^{-1}$, respectively) were about 1.3–4.0 times higher than the activities of *L. helveticus* M10 in the late-log phase of growth in milk (Figs. 3A and 4A). The highest activities were recorded when the cells were harvested in the late-log phase in both media. 18 out of 19 peptidase activities recorded in CFEs during *L. casei* RP0 growth in milk, and 12 peptidase activities in CFEs during growth in MRS broth showed the highest levels in the late-log phase (Fig. 4). 16 peptidase activities of *L. helveticus* M10 during growth in milk, and 15 activities during growth in MRS broth were the

highest in the late-log phase (Fig. 3). The activity of the proline-specific peptidases of the lactobacilli, attacking Pro-Gly-Gly, Arg-Pro-Pro and bradykinin, is independent of the growth phase at a later stage (after the mid-log phase) of cell growth in MRS broth (Figs. 3B, 4B and 6B).

All the 19 peptidase activities of *S. thermophilus* TEP4 recorded during growth in milk and 16 activities in M17 broth were significantly ($P < 0.05$) influenced by the growth phase (Fig. 5). 18 activities of *S. thermophilus* TEP4 during growth in milk and 13 activities in M17 broth showed the highest level

in the late-log phase of cell growth. The peptidase activity, hydrolyzing Pro-Gly-Gly, was not detected in CFEs after growth of *S. thermophilus* TEP4 in M17 broth (Fig. 5B). *S. thermophilus* TEP4 showed the highest specificity in the late-log phase to the dipeptides Leu-Gly ($210.34 \pm 7.78 \mu\text{mol}\cdot\text{mg protein}^{-1}$), Leu-Tyr ($266.40 \pm 4.10 \mu\text{mol}\cdot\text{mg protein}^{-1}$), Glu-Glu ($315.61 \pm 8.70 \mu\text{mol}\cdot\text{mg protein}^{-1}$) and Pro-Leu ($221.17 \pm 6.11 \mu\text{mol}\cdot\text{mg protein}^{-1}$), and to Ala-Pro- ρ Na ($188.43 \pm 7.37 \mu\text{mol}\cdot\text{mg protein}^{-1}$) (Fig. 5A).

3.2. Growth medium effect on the peptidase activities of *L. delbrueckii* ssp. *bulgaricus* 2-11, *L. helveticus* M10, *L. casei* ssp. *casei* RP0 and *S. thermophilus* TEP4

A comparison of the strains' peptidase activities, irrespective of the growth phase, revealed that about 80%–95% of the activities were significantly ($P < 0.05$) influenced by the growth medium (Figs. 2–6). 15 tested peptidase activities of *L. bulgaricus* 2-11 (6 aminopeptidases, 4 dipeptidases, 5 proline-specific peptidases and 1 oligopeptidase) were significantly ($P < 0.05$) higher during growth of *L. bulgaricus* 2-11 in milk than in MRS broth (Fig. 2). The peptidase, hydrolyzing Leu-Leu-Leu and proline-specific peptidases, hydrolyzing Pro-Leu, Pro-Gly-Gly and Pro-Leu-Gly-Gly, showed a higher level of specific activity during cell growth in MRS broth ($144.23 \pm 3.09 \text{ nmol}\cdot\text{mg protein}^{-1}$; $296.44 \pm 14.10 \mu\text{mol}\cdot\text{mg protein}^{-1}$; $0.48 \pm 0.51 \text{ nmol}\cdot\text{mg protein}^{-1}$ and $12.36 \pm 1.78 \text{ nmol}\cdot\text{mg protein}^{-1}$, respectively) (Fig. 2B).

The comparison of the highest specific activities recorded for each substrate for each growth medium, irrespective of the growth phase, showed that 18 activities of *L. casei* RP0 and 18 activities

of *L. helveticus* M10 were significantly dependent on the growth medium and had considerably higher levels ($P < 0.05$) when the cells were grown in milk (Figs. 3A and 4A). All tested peptidases belonged here except proline-specific peptidases, hydrolyzing Pro-Gly-Gly (*L. casei* RP0) and Pro-Leu-Gly-Gly (*L. helveticus* M10), which showed higher activity when the cells of both strains were grown in MRS broth (Figs. 3B and 4B).

The aminopeptidase activities were about 1.3–4 times (*L. helveticus* M10) and 2–6 times (*L. casei* RP0) higher in CFEs obtained from cells grown in milk compared with the cells grown in MRS broth (Figs. 3 and 4).

The comparison of the specific activities of *S. thermophilus* TEP4 showed that all peptidase activities were dependent on the growth medium, 17 late-log-phase peptidase activities, 16 mid-log-phase activities, and 14 stationary-phase activities being higher during growth in milk by about 1.5–4.5 times, 1.3–4 times and 1.2–2 times, respectively, than during growth in M17 broth. The aminopeptidases, hydrolyzing Leu- ρ Na, Met- ρ Na, Glu- ρ Na, Leu-Leu-Leu and Leu-Pro were an exception and showed higher specific activities during growth in M17 broth (Fig. 5B).

4. DISCUSSION

Hydrolysis of diagnostic substrates through cell lysates showed the presence of a wide range of intracellular (amino-, di-, tri- and oligopeptidases) peptidases in the CFEs of the selected proteolytic strains of lactobacilli and thermophilic streptococci. While there are reports in the literature dealing with peptidase activity in various strains of lactic acid bacteria, direct comparison between the data is difficult as the format for reporting enzyme activity is not standardized. The enzyme activities

are reported as presence or absence [55], as the mean specific activity [42, 54], as relative activity [31], as ratios [24], as activity against ρ -nitroanilide (ρ NA) substrates [45], and as activity against 7-amino-4-methylcoumarin (ACM) substrates [21]. Based on analysis of the peptidase activities, the strains *L. casei* RPO and *L. helveticus* M10 can be defined as highly proteolytic toward a wide range of substrates. The *L. casei* RPO strain possessed strong aminopeptidases, hydrolyzing Lys- ρ Na, Leu- ρ Na and Arg- ρ Na; dipeptidases, hydrolyzing Leu-Gly, Ala-His and Leu-Tyr; tripeptidase, hydrolyzing Leu-Leu-Leu, and proline-specific peptidases, hydrolyzing Ala-Pro- ρ NA, Leu-Pro, Pro-Leu and Arg-Pro-Pro (Fig. 4). *L. helveticus* M10 showed lower levels of peptidase activities, hydrolyzing Leu-Gly, Ala-Pro- ρ Na and Leu-Pro (Fig. 3). These results contradict earlier studies which present *L. helveticus* as by far the most proteolytic species [13, 21, 28, 42]. There are few reports on *L. casei* strains with higher levels of peptidolytic activity than *L. helveticus* [11, 53]. The relatively high level of total aminopeptidase activity obtained here supports reported enzyme activities for *L. helveticus* [42]. The data obtained for the proline-specific peptidases of *L. helveticus* M10, actively hydrolyzing the tested peptides, were not in accordance with the reported results for *L. helveticus* DPC4571 [21]. The results showed that both lactobacilli (*L. casei* RPO and *L. helveticus* M10) possess very efficient proteolytic systems, including the major aminopeptidases PepN and PepC, PepQ and PepR, endopeptidase and dipeptidases hydrolyzing Leu-Gly, Ala-His and Leu-Tyr. Despite the fact that all lactobacilli demonstrated high aminopeptidase activity, according to some authors, there is a possibility that the release of a chromogenic group could be a result not only of activity of the major aminopeptidases

PepN, PepC, and X-prolyl-dipeptidyl aminopeptidase (PepX), but also of the endopeptidase-proteinase activity.

The most striking difference between the levels of peptidase activities of the lactobacilli and *S. thermophilus* TEP4 was registered towards Glu-Glu hydrolysis. That substrate was efficiently hydrolyzed by *S. thermophilus* TEP4 (Fig. 5). Glutamyl aminopeptidase of *S. thermophilus* TEP4 (PepA) was most probably responsible for active hydrolysis of that substrate, which has also been established by other authors in *S. thermophilus* [39], and it is possible for an insignificant amount of that enzyme to be present in the *Lactobacillus* strains. In that case the hydrolysis of Glu-Glu and Glu- ρ Na with the *Lactobacillus* strains may not be caused by glutamyl aminopeptidase but rather by the total aminopeptidase activity. As for the four recorded proline-specific peptidases in *S. thermophilus* TEP4, the present results support the data given by Rul and Monnet [39]. Firstly, a high level of peptidase activity hydrolyzing Ala-Pro- ρ Na was found. Secondly, a peptidase which hydrolyzes Arg-Pro-Pro was detected. Proline-specific dipeptidase was found – a high-level prolinase (PepR), attacking Pro-X dipeptides (Pro-Leu). Iminopeptidase activity (Pro- ρ Na) in CFEs of *S. thermophilus* TEP4 was established, which was not found in the *S. thermophilus* CNRZ strain by the above authors. Oligopeptidase activity (PepF) in *S. thermophilus* TEP4 was found using bradykinin as a substrate (Fig. 6), and the results are in unison with studies showing the presence of the PepF-type of oligopeptidase in *S. thermophilus* [40].

Because of the high content of proline in casein (11.7% in α_s -casein and 16.7% in β -casein), enzymes capable of degrading peptides containing proline are thought to be of the utmost importance for the degradation of casein by lactic acid bacteria. Some lactic acid bacteria depend on casein

as their sole source of proline, since they are auxotrophic for this amino acid [25]. The general aminopeptidases PepC and PepN from *L. lactis*, according to the authors, cannot release amino-terminal proline residues from peptides [50]. Proline iminopeptidase (PepI) of *L. lactis* is highly specific for di- and tripeptides with a N-terminal proline residue, unable to remove N-terminal Pro residue from tetrapeptides or Pro- ρ Na [25]. PepI does not hydrolyze peptides with Pro residue at the penultimate position. *Lactobacillus* strains and *S. thermophilus* TEP4 hydrolyzed chromogenic substrate Pro- ρ Na. Our studies proved the hydrolyzing action of PepT toward the Pro-Gly-Gly substrate, described by other authors for tripeptidase (PepT) from *L. lactis* ssp. *cremoris* Wg2 [25]. Lactococci seem to depend on endopeptidase activity and/or X-prolyl-dipeptidyl aminopeptidase (PepX) for utilizing proline-containing oligopeptides [25, 36]. The recorded high Ala-Pro- ρ Na hydrolysis activity of *L. casei* RP0 (810.33 $\mu\text{mol}\cdot\text{mg protein}^{-1}$), *L. bulgaricus* 2-11 (388.11 $\mu\text{mol}\cdot\text{mg protein}^{-1}$), *L. helveticus* M10 (274.44 $\mu\text{mol}\cdot\text{mg protein}^{-1}$) and *S. thermophilus* TEP4 (188.43 $\mu\text{mol}\cdot\text{mg protein}^{-1}$) suggests a considerable rate of release of dipeptides with penultimate or N-terminal proline residue from oligopeptides (Figs. 2–5). The peptidase activity of *L. helveticus* M10, hydrolyzing Ala-Pro- ρ Na, exceeded by about 5 times the level reported by other authors for *L. helveticus* strains [21, 42]. The results obtained do not support the studies according to which the growth medium and growth phase do not significantly influence the proline-specific peptidase activities, hydrolyzing Ala-Pro- ρ Na and Pro- ρ Na of two strains of *L. casei* grown in MRS [17]. These activities of *L. casei* RP0 were significantly ($P < 0.05$) influenced by the growth medium and growth phase, with the highest specific activities in the late-log phase during

growth in RSM (Fig. 4). In contrast to the measured high prolinase activity in CFE of *L. helveticus* M10, no prolinase activity was recorded in CFE of *L. helveticus* by other authors [22].

In CFEs of all bacterial cultures oligopeptidase PepF, capable of hydrolyzing the long peptide bradykinin (9 amino acid residues), was found (Fig. 6). The Pro7-Phe8 bond of bradykinin was rapidly hydrolyzed, showing that the enzyme produced from the strains was efficient for the hydrolysis of prolyl peptide bonds. According to some authors, endopeptidase PepO, too, effectively hydrolyzes polypeptides (like bradykinin), but is unable to degrade di-, tri- and tetrapeptides [25]. Other authors hold the opinion that endopeptidase PepO is not essential for utilizing casein or oligopeptides [32, 51]. Obviously, endopeptidase activity can be replaced by other peptidases with similar substrate specificities.

The growth medium and growth phase in which the cells were obtained had a significant role in the specific activity of the peptidases of the *Lactobacillus* strains and thermophilic streptococci. The fact that over 90% of the peptidase activities were significantly higher in milk than in MRS/M17 broth emphasizes the importance of using milk as a growth medium in peptidase studies of starter cultures. The higher level of peptidase activities in CFEs obtained from cells grown in milk compared with MRS/M17, according to some authors, is consistent with the fact that milk has a low concentration of peptides and free amino acids [15, 29]. For that reason nutritional regulation of peptidase biosynthesis is likely to occur in milk at higher levels.

The available results about the growth phase at which the cells are harvested, and optimal levels of proteolytic activity are obtained, are controversial. A large number of enzyme studies have been carried out on cells growing in broth to the stationary

phase, and on frozen cells [15, 54, 55]. There are reports on cell harvesting in the mid-log phase [42], as well as in the late-log phase [42, 45]. A fact that cannot be ignored is that certain strains of a species have relatively large differences in the levels of proteolytic activity when the cells are harvested at the same growth phase, as well as the fact that growth in milk can influence the synthesis of some enzymes [45]. For cells grown in milk the peptidase activity was at a higher level in the late-log phase, compared with the mid-log phase and stationary phase; for cells grown in MRS/M17 the increased regulation was related to the late-log phase and to the stationary phase, with few exceptions (Figs. 2–5). These differences can be explained by nutritional regulation according to some authors [21]. The authors assume that in MRS/M17 broth, when the culture enters the late-log phase and stationary phase, the use of amino acids and peptides could eliminate the repression of peptidase biosynthesis, which leads to higher activities. According to them, the increased regulation of peptidase activity at a higher level in the late-log phase in milk agrees with the fact that the cells are harvested at the end of the exponential phase when the cell concentration reaches a maximum level; the higher concentrations of amino acids and peptides thus obtained cause repression at the beginning of the stationary phase.

5. CONCLUSIONS

The results give information on the intracellular peptidase activities for 4 strains of lactic acid bacteria, used as operating strains for manufacturing of Bulgarian yogurt and traditional Bulgarian cheeses (white-brined cheese and Kashkaval cheese). Ranking strains of lactic acid bacteria according to peptidase activity can be a way to select strains that possess enzyme potential to participate in starter cultures,

or a way of using lactobacilli as nonstarter cultures, which will complement the activities already available in the starter cultures, thus having an influence on the flavor formation during cheese ripening. The yogurt bacteria *L. bulgaricus* 2-11 and *S. thermophilus* TEP4 demonstrated the presence of strong aminopeptidases, dipeptidases and proline-specific peptidases. The strains *L. casei* RP0 and *L. helveticus* M10, used in starter cultures and as nonstarter (adjunct) cultures in white-brined cheese and Kashkaval cheese manufacture, revealed a highly developed peptidase system that is important for cheese ripening. In previous studies *L. casei* RP0, *L. bulgaricus* 2-11 and *L. helveticus* M10 strains were shown to undergo lysis during cheese ripening, releasing their intracellular enzymes, which, in turn, enhance proteolysis, accelerate ripening and improve cheese flavor [46].

The growth medium and growth phase at which the cells are harvested are factors with a significant effect on peptidase activities of CFEs of lactobacilli and thermophilic streptococci. The higher levels of peptidase activity in milk than in synthetic broth media (MRS/M17), regardless of growth phase, emphasize the importance of the natural growth medium when conducting peptidase studies of starter cultures. The higher level of peptidase activity of strains in the late-log phase indicates the advantage of that phase for cell harvesting in the preparation of CFEs for studying the peptidase potential of lactic acid bacteria.

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