Bulgarian Chemical Communications, Volume 55, Special Issue C (pp. 57–63) 2023 *DOI: 10.34049/bcc.55.C.0006*

Specific protease- and aminopeptidase activity of potential bioactive peptide-producing lactobacilli in media with plant protein hydrolysates

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Received: November 2023; Revised: December 2023

In this study selected strains of Lactobacillus, potential producers of bioactive peptides, were evaluated for their specific protease- and aminopeptidase activity after growth in milk, mMRS (MRS without peptone and meat extract) and mMRS with added pea protein hydrolysate, soy protein hydrolysate or whey protein 80 (WP 80). Five *Lactobacillus helveticus* strains (AB, h25, h48, h70, b244) and *Lacticaseibacillus casei* c1 (LB Bulgaricum PLC, Sofia, Bulgaria) were assayed. With minor exceptions the protease activity varied between strains to a much larger extent (> 10 fold) than it was influenced by the composition of each growth medium (up to 3 fold). This makes the selection of a highly proteolytic strains of primary importance. Although mMRS composition differed largely from milk, the protease activity of cells grown in mMRS remained high. Leucine- and lysine-aminopeptidase activities were highest in milk and mMRS, while as a rule the addition of plant hydrolysates or WP 80 resulted in lower values. As a whole the activities of these two aminopeptidases followed a constitutive pattern. On the contrary the arginine- and proline-aminopeptidase activities showed inducible character with measurable values obtained only in milk and mMRS with added pea protein hydrolysate. In the end of the study we selected two *L. helveticus* strains (b244 and h70) with high protease and aminopeptidase activity for further experiments.

Keywords: aminopeptidase activity, lactobacilli, plant protein hydrolysates, protease activity

INTRODUCTION

Products obtained after fermentation with selected lactobacilli can improve health, while dairy products are widely present in the daily life of every family. However, people suffering from allergy to milk proteins or lactose intolerance are recommended to consume alternative products [1]. A worldwide trend is the growing popularity of plant-based products [2]. On the other hand, with the exception of soy-products, alternative milk substituents and plant-based products contain less protein than dairy products [3, 4]. Also, plant proteins are more difficult for the human body to digest due to the presence of antinutritional factor compounds such as protease inhibitors and non-starch polysaccharides, as well as properties inherent to the protein structure such as cross-linking, hydrophobicity and secondary structure elements [5].

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Pea and soy proteins are some of the most commonly used plant proteins for the production of dairy alternatives. The main components in pea (*Pisum sativum*) are protein (20–25%), fat (1.5–2.0%), starch (24–49%) and dietary fibers (60–65%), while vitamins, minerals, phytic acid, saponins, polyphenols, and oxalates are present as minor constituents [6]. The basic mineral elements contained in pea are potassium (1.04%), phosphorous (0.39%), magnesium (0.10%), and calcium (0.08%). Pea preparations also provide a large quantity of water-soluble vitamins, especially B-group vitamins, as well as essential amino acids such as lysine and threonine [7].

Unlike most legumes, soybeans have high protein content, making soybeans and their food derivatives excellent plant-based protein sources [8]. Soybeans contain protein (35–40%), lipids (20%) and dietary fibers (9%) based on the dry weight of mature raw seeds [9]. Soy and pea proteins are rich in sulphur-containing amino acids. According to Qin *et al.* [10] digestibility of these proteins is 95–98% and 83–90% for soy and pea, respectively.

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Lactic acid bacteria (LAB) are one of the most well-studied microorganisms. Certain health-promoting properties of LAB strains are related to the production of bioactive peptides. These are well studied in milk media, but there are less reports of LAB-derived peptides in plant-based products. Bioactive peptides are final products of the activity of proteinases, peptidases and specific transport systems, all components of the proteolytic system of LAB [11, 12] (Fig. 1). All of these enzyme activities and cell functions are influenced by the composition of the growth medium and growth conditions. The present study investigates the effect of semi-defined growth media, containing pea or soy-protein hydrolysates, compared to milk on the specific protease activity and several aminopeptidase activities in cell preparations from strains of lactobacilli with potential to produce bioactive peptides.

MATERIALS AND METHODS

Five *Lactobacillus helveticus* strains (AB, h25, h48, h70, b244) and one *Lacticaseibacillus casei* (c1) all maintained in the LBB Culture Collection of LB Bulgaricum PLC, Sofia, Bulgaria, were used in the study. The selected lactobacilli were cultured for 16 hours at 37 °C in sterile 10% reconstituted skimmed milk (RSM), mMRS (containing glucose, Tween 80, sodium citrate, sodium-acetate, di-potassium hydrogen phosphate trihydrate, yeast extract, magnesium and manganese salts) and mMRS with added 1% pea protein hydrolysate (PPH), mMRS with 1% soy protein hydrolysate (SPH) and mMRS with 1% whey protein 80 (WP 80).

The pH of the strains was measured after 16 hours of cultivation. The number of microorganisms in milk medium was determined after plating suitable decimal dilutions on MRS agar and anaerobic incubation for 48 hours at 37 °C. Growth of bacteria in mMRS-based media was evaluated spectrophotometrically by measuring the optical density at 600 nm against uninoculated media. Then cells were harvested by centrifugation, washed three times with phosphate buffer (pH 7,5) and disrupted by sonification.

The specific protease activity (PA_{sp}) was determined with the Enzymatic Assay of protease casein as a substrate (SSCASE01.001, 04.02.99) method [13]. Protease activity (PA) was defined as the amount of enzyme that catalyzes the release of 1 μmol of tyrosine for 1 minute at pH 7,5 and 37 °C. PA was expressed in U/ml. PA_{sp} was calculated as the ratio of PA and the protein content in the sample determined by the Bradford method [14].

In order to assess aminopeptidase activity (APA) we used four chromogenic substrates L-leucine-p-

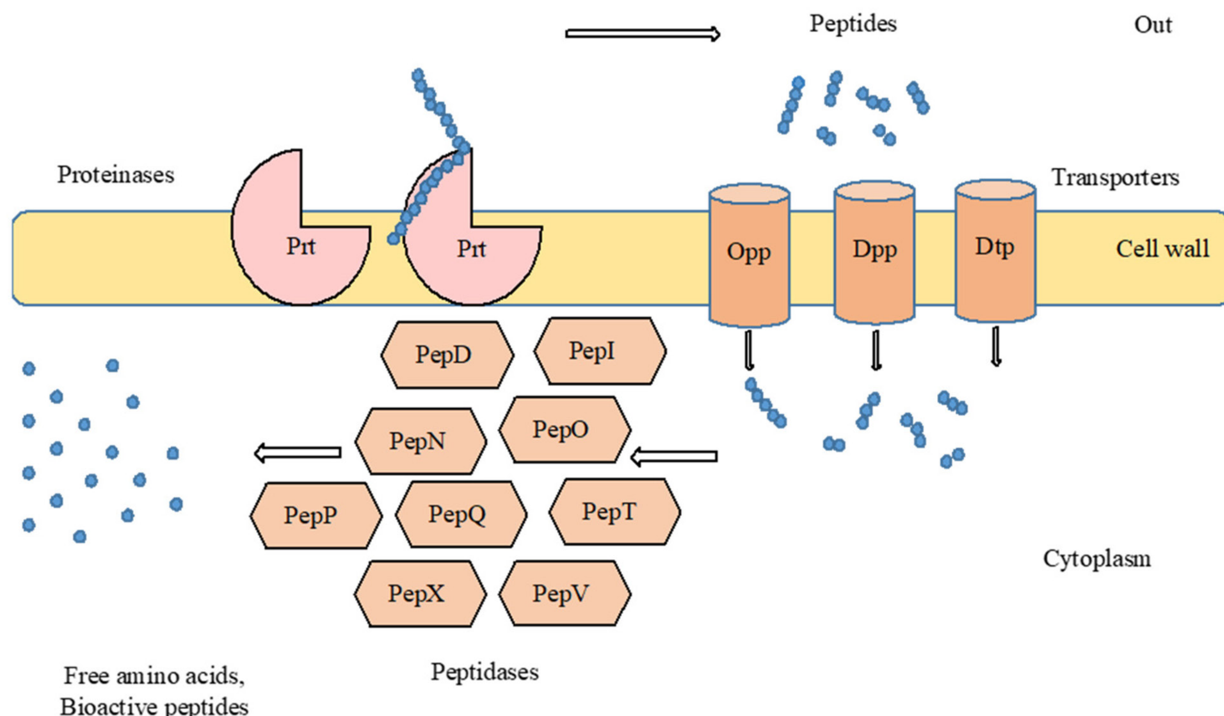

Fig. 1. Schematic representation of the proteolytic system of *Lactobacillus* ssp.

nitroanilide (L-leu-pNA), L-lysine-p-nitroanilide (L-lys-pNA), L-arginine-β-naphthylamide (L-arg-βNA) and L-proline-β-naphthylamide (L-pro-βNA) [15,16]. APA for the first two substrates was calculated using the formula:

$$\text{APA (pNA)} = \frac{(\Delta\text{A405 nm Test Sample} - \Delta\text{A405 nm Blank})(3)\text{df}}{(10{,}8)(0{,}1)} \quad (1)$$

where: ΔA_{405} nm Test Sample/Blank – maximal linear change in absorption at 405 nm per minute for the Test Sample or the Blank for a measurement period of 5 minutes; 3 – Total volume, ml; df – Dilution factor; 10,8 – Millimolar extinction coefficient of p-Nitroanilide at A405nm, $M^{-1}cm^{-1}$; 0,1 – Sample volume, ml.

The calculation of APA for the second two substrates was different. The APA of β-naphthylamides was measured indirectly by the reaction of released β-naphthylamines with a stabilized diazonium salt, Fast Garnet GBC and Brij35, producing a red azo dye which was evaluated spectrophotometrically at 550 nm. Absorptions were measured at the beginning (0 min) and at the end (60 min) of the enzymatic reaction. APA for the L-arg-βNA and L-pro-βNA was calculated using this formula:

$$\text{APA }(\beta\text{NA}) = \frac{(\Delta\text{A550 nm})/(60)(5)}{(20)(0{,}1)} \quad (2)$$

where: $\Delta A_{550\ nm}$ – Change in absorption at 550 nm for a measurement period of 60 minutes; 60 min – Duration of enzyme reaction; 5 – Total volume, ml; 20 – Millimolar extinction coefficient of β-Naphthylamide at A550 nm, $M^{-1}cm^{-1}$; 0,1 – Sample volume, ml.

The specific APA (APA_{sp}) for all four substrates was calculated as a ratio between APA and the protein content.

RESULTS AND DISCUSSION

Growth of the cultures in the tested media

After 16 hours of cultivation, the strains showed a normal acidification process in all tested media. All strains with the exception of *Lc. casei* c1 coagulated RSM with a final pH in the range of 3,51–4,34 (pH for *Lc. casei* was 5,13). Viable cell counts in RSM for all strains were of the same order (1 $\times 10^8$–7,8 $\times 10^8$ cfu/ml). In mMRS-derived media the final pH was in the interval of 3,5–4,0. The final optical density of the cultures was variable and strain-specific (Fig. 2). The addition of plant protein hydrolysates and whey protein (WP80) to mMRS had no significant effect on the measured final pH and optical density values.

Specific protease activity (PA_{sp})

The PA_{sp} of cells grown on different media was strictly strain-specific (Fig. 3). Regarding the different types of media, the highest values were observed in milk medium and mMRS without additives – 64,96 and 57,29 U/mg, respectively. The highest PA_{sp} values in RSM were measured with *L. helveticus* h70 followed by b244 and h25. Interestingly, all *L. helveticus* strains had comparable or higher PA_{sp} values in mMRS compared to RSM or mMRS media with additives. In mMRS *L. helveticus* b244

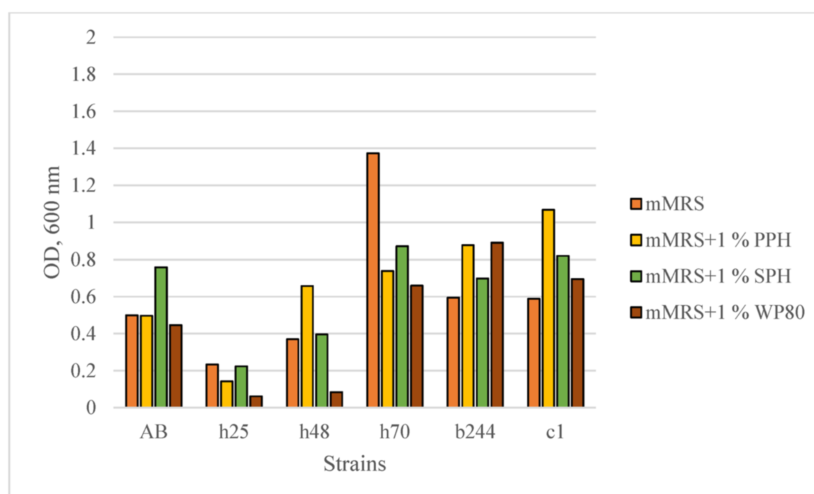

Fig. 2. Optical density in different growth media: mMRS – modified MRS without peptone and meat extract; PPH – pea protein hydrolysate; WP 80 – whey protein; SPH – soy protein hydrolysate * AB, h25, h48, h70, b244, c1 – LAB strains.

showed the highest PA_{sp} value followed by h70, h25 and h48. As a rule, the addition of plant protein hydrolysates reduced the protease activity for the most of the *L. helveticus* strains. For example, the addition of PPH to mMRS resulted in a 5-6-fold reduction in the PA_{sp} values compared to RSM and mMRS. Strain h25 had negligible PA_{sp} values on all mMRS media with additives. A notable exception was strain b244 with comparable PA_{sp} values on all tested media. Interestingly in mMRS with 1% PPH AB and h48 strains showed higher results compared to RSM and mMRS. As in the case of mMRS-PPH, mMRS with added SPH resulted in low PA_{sp} values of the *L. helveticus* cells. The addition of WP 80 to mMRS produced cells with PA_{sp} comparable to RSM and mMRS only for strains h70 and b244 – 30,26 U/mg and 53,71 U/mg, respectively. For other *L. helveticus* strains cultured in mMRS with WP 80 the values were minor. Compared to the *L. helveticus* strains, *Lc. casei* c1 showed low, but constant protease activity on all mMRS-derived media (not determined in milk). *L. helveticus* h70 and b244 proved to be the strongest proteolytic strains in the tested group.

Specific aminopeptidase activity (APA_{sp})

APA_{sp} with L-leu-pNA and L-lys-pNA as substrates

The results for APA_{sp} with L-leu-pNA and L-lys-pNA as substrates varied greatly between strains and growth media. For cells grown in RSM, the highest results were obtained for *L. helveticus* strains h70, h25 and b244 with values for the two substrates (L-leu-pNA/L-lys-pNA) of 350/344 U/mg, 299/356 U/mg and 224/195 U/mg, respectively (Fig. 4A). *L. helveticus* AB had negligible APA_{sp} for L-leu-pNA and L-lys-pNA in RSM, while RSM was not suitable for growth of *Lc. casei* c1.

Growth in mMRS without any additives resulted in cells with the highest APA_{sp} for L-leu-pNA and L-lys-pNA among all tested media (Fig. 4B). Values for all strains were significantly higher compared to RSM. This was especially well shown for *L. helveticus* strain h25 reached APA_{sp} of 696 U/mg and 816 U/mg for L-leu-pNA and L-lys-pNA, respectively. Unlike results for RSM, cells of *L. helveticus* AB grown in mMRS had APA_{sp} comparable to other tested *L. helveticus* strains. In mMRS *Lc. casei* c1 had detectable, although lower APA_{sp} compared to *L. helveticus* strains for L-leu-pNA and L-lys-pNA (Fig. 4B).

Similar to the observed decrease in the specific protease activity, the addition of plant protein hydrolysates to mMRS resulted in significant reduction in APA_{sp} measured with L-leu-pNA and L-lys-pNA (Figure 4C, D). In mMRS with 1% PPH APA_{sp} values varied between 28 to 119 U/mg and from 32 to 189 U/mg for L-leu-pNA and L-lys-pNA, respectively, with values for strains AB, h48, h70 and b244 being of the same order (Fig. 4C). Similar results were obtained in mMRS with 1 % SPH where for the cells of *L. helveticus* strains AB, h70 and b244 the measured APA_{sp} for the two substrates (L-

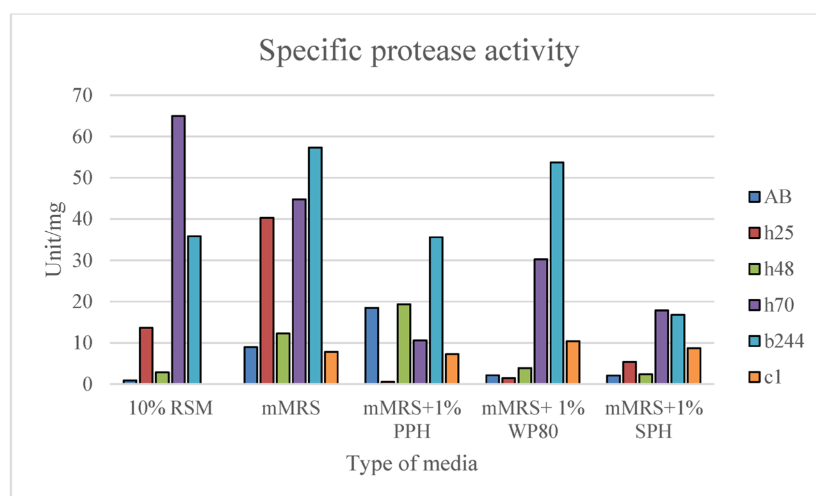

Fig. 3. Specific protease activity (PA_{sp})of cells grown in different growth media: RSM – 10 % reconstituted skimmed milk; mMRS – modified MRS without peptone and meat extract; PPH – pea protein hydrolysate; WP 80 – whey protein; SPH – soy protein hydrolysate
* AB, h25, h48, h70, b244, c1 – LAB strains.

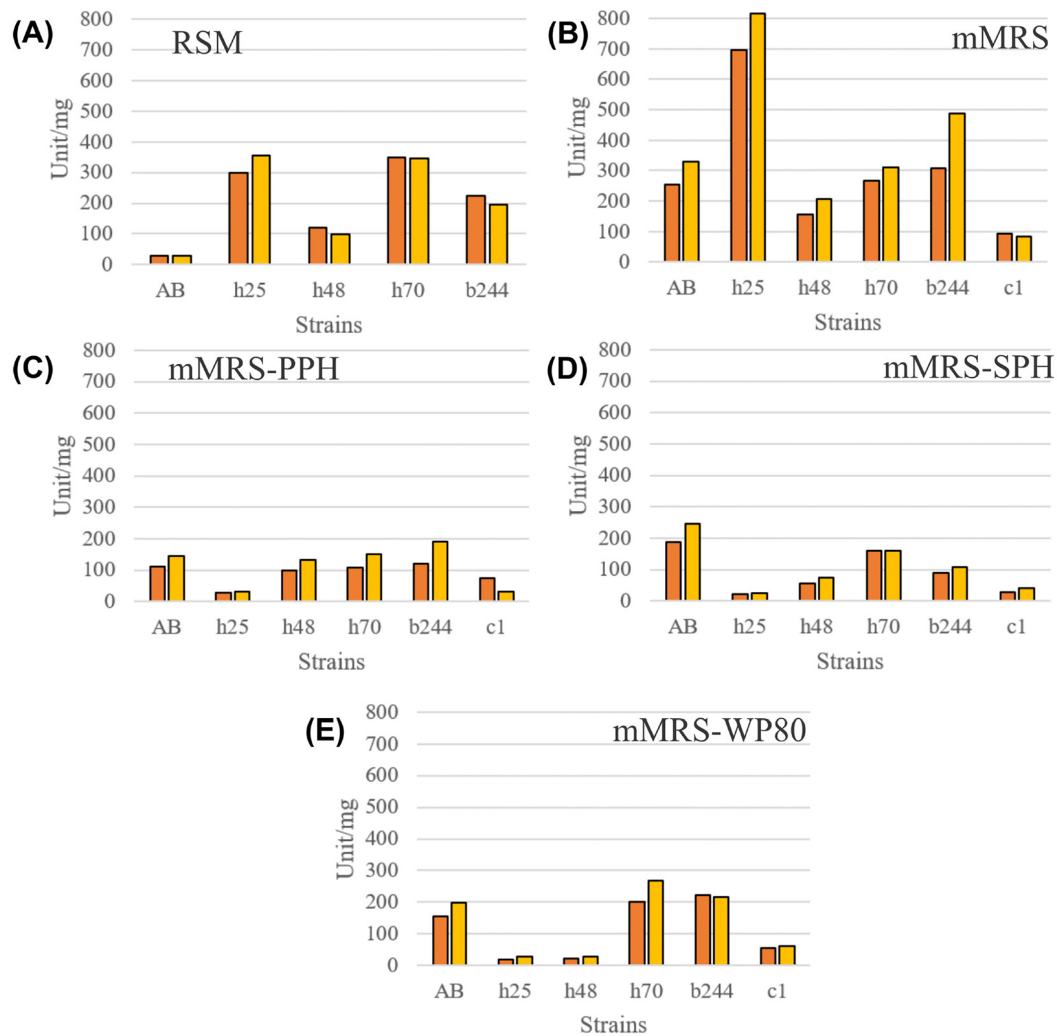

Fig. 4. Specific aminopeptidase activity (APA_{sp}) of cells grown in different media with L-leucine-p-nitroanilide (■) and L-lysine-p-nitroanilide (■) as substrates.

leu-pNA/L-lys-pNA) was 186/246 U/mg, 159/159 U/mg and 89/107 U/mg, respectively (Fig. 4D).

The results for strains cultivated in mMRS with WP 80 are shown in Fig. 4E. The APA_{sp} of cells grown in this medium were slightly higher than mMRS-PPH and mMRS-SPH media but still substantially below the high values measured for mMRS without additives. Cells of *L. helveticus* strains h70, b244 and AB harvested from mMRS-WP80 showed APA_{sp} values for the two substrates (L-leu-pNA/L-lys-pNA) of 201/269 U/mg, 222/215 U/mg and 154/196 U/mg, respectively (Fig. 4E).

On all mMRS media with or without the additives *Lc. casei* c1 showed low, but constant APA_{sp} for these two substrates (Fig. 4B-E). From the point of view of selection of strains with high APA_{sp} for L-leu-pNA and L-lys-pNA the best performing strains on all tested media were *L. helveticus* strains h70 and b244.

Notably, there was a clear correlation between the APA_{sp} values measured separately for L-leu-pNA and L-lys-pNA with all tested cultures (Fig. 4A-E). This might suggest that these two enzymatic activities are closely related, overlapping in substrate specificity and/or regulated in an identical manner.

APA_{sp} with L-arg- βNA and L-pro- βNA as substrates

Although units of specific enzyme activity for APA with the four chromogenic substrates were defined in an identical manner, values of APA_{sp} for the L-arg-βNA and L-pro-βNA substrates were of

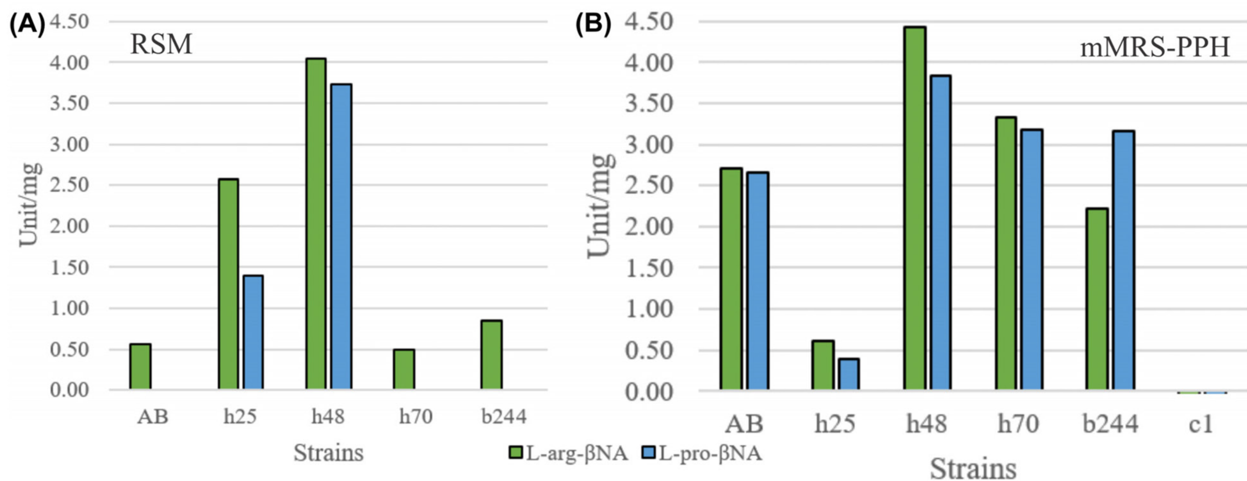

Fig. 5. (A–B) Specific aminopeptidase activity (APA_{sp}) of cells with L-arginine-β-naphthylamide and L-proline-β-naphthylamide substrates.

significantly lower scale compared to L-leu-pNA and L-lys-pNA (Fig. 5A, B). Moreover, detectable activity towards L-arg-βNA and L-pro-βNA was only measured in RSM and mMRS with addition of 1% PPH. Values of APA_{sp} for L-arg-βNA and L-pro-βNA were strain-specific with higher activity (> 1U/mg) measured for two of the five tested *L. helveticus* strains (h25 and h48) in RSM and for four of the same five cultures (AB, h48, h70 and b244) in mMRS-PPH. Cells of *Lc. casei* c1 showed no activity towards L-arg-βNA and L-pro-βNA on all tested media.

Unlike APA measured with L-leu-pNA and L-lys-pNA, activity towards the L-arg-βNA and L-pro-βNA substrates was practically very low or undetectable for the majority of strains and tested media (mMRS, mMRS-SPH, mMRS-WP80). These results suggest that peptidase activity for peptides with arginine or proline at their N-terminus is tightly regulated and specific for the composition of the growth medium, possibly related to the availability of these two amino-acids in the medium.

Values of specific protease activity and APA_{sp} for L-leu-pNA and L-lys-pNA both showed negative correlation with the addition of plant protein hydrolysates and WP80. The ready availability of low-molecular nitrogen source in the growth medium (peptides from protein hydrolysates) seems to lower the pressure on the proteolytic system of the culture, resulting in lower values of the tested enzyme activities.

CONCLUSIONS

The highest levels of specific protease activity were observed in mMRS, comparable to RSM, while lower values were obtained with the addition of plant protein hydrolysates and WP80. The response to the growth medium of each culture with respect to specific aminopeptidase activity with L-leu-pNA and L-lys-pNA substrates was strain-dependent with highest values in mMRS and RSM and a decrease with the addition of plant protein hydrolysates and WP80 to the medium. Specific aminopeptidase activity with L-arg-βNA and L-pro-βNA as substrates was barely detectable and only in RSM and mMRS with added PPH. The best performing strains with constant protease and aminopeptidase activity on all tested media were *L. helveticus* h70 and b244.

Acknowledgements: *This participation is financed by the operational program "Science and education for smart growth" 2014–2020, grant number BG-05M2OP001-1.002-0005-C01, Personalized Innovative Medicine Competence Center (PERIMED).*

REFERENCES

1. M. Montemurro,E. Pontonio, R. Coda, C. G. Rizzello, *Foods*, **10**, 316 (2021).
2. S. Hertzler, J. C. Lieblein-Boff , M. Weiler, C. Allgeier, *Nutrients*, **12**, 3704 (2020).

3. O. E. Mäkinen, V. Wanhalinna, E. Zannini, E. K. Arendt, *Crit. Rev. Food Sci. Nutr.*, **56**(3), 339 (2016).
4. M. E. Clegg, A. T. Ribes, R. Reynolds, K. Kliem, S. Stergiadis, *Food Research International*, **148**, 110586 (2021).
5. A. G. A. Sá, Y. M. F. Moreno, B. A. M. Carciofi, *Crit. Rev. Food Sci. Nutr.*, **60**, 3367 (2020).
6. P. Shanthakumar, J. Klepacka, A. Bains, P. Chawla, S. B. Dhull, A. Najda, *Molecules*, **27**, 5354 (2022).
7. K.A. Millar, E. Gallagherb, R. Burkec, S. McCarthyd, C. Barry-Ryan, *J. Food Compos. Anal*., **82**, 103233 (2019).
8. C. Chatterjee, S. Gleddie, C. W. Xiao, *Nutrients*, **10**, 1211 (2018).
9. A. J. Michelfelder, *Am. Fam. Physician*, **79**, 43 (2009).
10. P. Qin, T. Wang, Y. Luo, *J. Agri. Food Research*, **7**, 100265 (2022).
11. C. Raveschot, B. Cudennec, F. Coutte, C. Flahaut, M. Fremont, D. Drider, P. Dhulster, *Frontiers Micro.*, **9**, (2018).
12. M. Kieliszek, K. Pobiega, K. Piwowarek, A. M. Kot, *Molecules*, **26**, 1858 (2021).
13. O. Folin, V. Ciocalteu, *J. Biol. Chemistry*, **73**, 627 (1927).
14. M. Bradford, *Analytical Biochemistry*, **72**(1–2), 248 (1976).
15. M. Sasaki, B. W. Bosman, P. S. Tan, *J. Dairy Research*, **62**, 601 (1995).
16. T. C. Eleman, *Biochem. J*., **141**, 113 (1974).