Appl Biochem Biotechnol (2011) 163:994–1001
DOI 10.1007/s12010-010-9103-0

Proline-Specific Extracellular Aminopeptidase Purified from *Streptomyces lavendulae*

Arya Nandan · Ashok Pandey · Kesavan Madhavan Nampoothiri

Received: 28 June 2010 / Accepted: 29 September 2010 /
Published online: 13 October 2010

Abstract Aminopeptidases catalyze the cleavage of specific amino acids from the amino terminus of protein or peptide substrates. A proline-specific aminopeptidase was purified to homogeneity from the culture-free extract of *Streptomyces lavendulae* ATCC 14162 in sequential steps comprising ammonium sulfate precipitation, ultra-filtration, and column chromatography on Q-sepharose and Sephadex G-100. The purified protein showed approximately 60 kDa in SDS-PAGE and was optimally active at pH 6.5 and 40 °C. Kinetic studies showed a K_m and V_{max} of 0.23 mM and 0.087 μmol/min, respectively, using Pro-*p*-NA, the substrate with maximum specificity. Enzyme activity was inhibited by PMSF and ions like Zn^{2+}, Co^{2+}, and Ni^{2+}. However, unlike other aminopeptidases, the activity was enhanced in the presence of DTT, 1,10-phenanthroline, EDTA, amastatin, and bestatin. Ions like Ca^{2+}, Mg^{2+}, and Mn^{2+} also enhanced the activity.

Keywords L-Proline aminopeptidase · L-Proline *p*-nitroanilide · Serine protease · *Streptomyces lavendulae*

Introduction

Aminopeptidases form a significant enzyme family [1] and are associated with many biological functions such as protein degradation and protein maturation [2]. In the food industry, bacterial enzymes are valuable for the preparation of protein hydrolysates such as casein, collagen, and gluten hydrolysates that are used as food ingredients and supplements [3]. Broad-specificity aminopeptidases can release most N-terminal amino acids from peptides; however, they are unable to cleave imido bonds and consequently cannot release N-terminal amino acids when the penultimate residue is proline [4, 5]. Proline

A. Nandan · A. Pandey · K. M. Nampoothiri (✉)
Biotechnology Division, National Institute for Interdisciplinary Science and Technology (NIIST), CSIR, Trivandrum, Thiruvananthapuram 695 019 Kerala, India
e-mail: madhavan85@hotmail.com

K. M. Nampoothiri
e-mail: m.nampoothiri@rediffmail.com

aminopeptidase (PAP) is therefore required to remove the proline residues and is essential for the degradation of proline-rich peptides and proteins, such as collagen and gelatin [6]. The crystal structure and catalytic mechanism of the proline aminopeptidase from *Escherichia coli* has been reported [7, 8]. The genus *Streptomyces* was proved to be a good source of various substrate-specific aminopeptidases such as leucine or methionine aminopeptidase [9, 10]. The aminopeptidases from *Streptomyces* are of particular interest for biochemical and biomedical applications as they are stable and have a low molecular weight, simple kinetics, and a high enzyme activity [11]. Hence, an attempt was made to screen various *Streptomyces* strains for proline-specific aminopeptidase activity.

Materials and Methods

Materials

Substrates like L-Pro-*p*Na, L-Leu-*p*Na, L-Arg-*p*NA, L-Met-*p*NA, L-Gly-*p*NA, and L-Val-*p*NA, protease inhibitors, and chromatography resins were obtained from Sigma (USA). Media components and metal ions were procured from Himedia (Mumbai, India). All other chemicals are of analytical grade and obtained from Genei (Bangalore, India).

Microorganism and Maintenance

Streptomyces lavendulae ATCC 14162 was subcultured in the ATCC-5 medium containing (% *w*/*v*): beef extract, 0.1; yeast extract, 0.1; tryptone, 0.2; glucose, 1; $FeSO_4$, 0.001; agar, 2; and pH 6.8. The plates were incubated at 30 °C and the fully sporulated plates (4 days) were kept at 4 °C; glycerol stocks (50%) were made for long-time preservation.

Inoculum and Fermentation

One loopful of the culture was transferred to 20 mL ATCC-5 medium (without agar) in 50 mL Erlenmeyer flasks. The inoculated flasks were incubated in a rotary shaker at 30 °C at 200 rpm for 24 h. Only 1% *v*/*v* (9×10^4 CFU/mL) of the above culture was used as inoculum. Fermentation for PAP production was carried out in 250 mL Erlenmeyer flasks containing 100 mL of YEME medium (% *w*/*v*): yeast extract, 0.3; malt extract, 0.5; bacto peptone, 0.3; and lactose, 1; and pH 6.8. The flasks were incubated as mentioned earlier and the samples were withdrawn at the desired interval of time.

Enzyme Assay

The reaction mixture contained 2.5 mML-Proline *p*-nitroanilide, 50 mM Tris HCl buffer (pH 8.5), and 50 μL enzyme sample and the assay was done as per the protocol described by Tan and Konings [12] on 96-well plates using a microplate reader (Model 680XR, Bio-Rad, USA). One IU of enzyme activity was defined as the amount of enzyme that hydrolyses 1 μM of L-Pro *p*-nitroanilide per minute.

Enzyme Purification

A total of 500 mL of cell-free culture filtrate was used for ammonium sulfate fractionation to a final concentration of 80%. The fractions with maximum specific activity were pooled

and were ultra-filtered using 30 kDa Amicon membrane filters. The fraction above 30 kDa was collected and used for Ion Exchange Chromatography in Q-Sepharose column (1.6× 20 cm, Amersham Biosciences, UK) which is pre-equilibrated with 50 mM Tris HCl buffer pH 7. After washing with the same buffer, the proteins were eluted with 100 mL linear gradient of 0–0.5 M NaCl in 50 mM Tris HCl buffer at a flow rate of 1 mL/min. The active fractions were pooled, concentrated by lyophilization, and dialyzed against 50 mM Tris HCl buffer, pH 7 at 4 °C. The pooled active fraction from this step was subjected to gel filtration on Sephadex G-100 column (1.6×80 cm, Amersham Biosciences, UK), previously equilibrated with 50 mM Tris HCl buffer pH 8.5. Proteins were eluted at 0.5 mL/min and collected as 1 mL fractions. The active fractions were pooled and were concentrated by lyophilization and stored at −80 °C.

SDS–PAGE and Activity Staining

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 12%) [13] was performed to analyze the different purified fractions. For activity staining, the protein was first separated on 10% Native PAGE and the procedure described by Bozic and Vujcic [14] was followed. Briefly, the gel was washed twice with distilled water for 10 min followed by 50 mM Tris–HCl buffer, pH 8.5, twice for 5 min. The gel was dipped into 1 mM L-proline *p*-nitroanilide and incubated at 37 °C for 20 min. The diazotization of liberated *p*-nitroanilide was done by immersing in 0.1% (*w*/*v*) $NaNO_2$ in 1 N HCl for 2 min. The excess $NaNO_2$ was removed by washing with 1% (*w*/*v*) urea. The gel was finally immersed in 0.025% (*w*/*v*) 1-napthylamine solution in 22% ethanol until the pink azo dye was formed.

Biochemical Characterization

Effect of pH and Temperature

The PAP activity was assayed against Pro-*p*NA at varying pH range from 3 to 10.5 using different buffer systems such as 100 mM acetate buffer (pH 3 to 6), 100 mM phosphate buffer (pH 6 to 7), 100 mM Tris HCl buffer (pH 7 to 9), and 100 mM sodium glycine buffer (pH 9.5 to 10.5). The results were expressed as the percentage of the activity obtained compared to the activity at the optimum pH.

The temperature optimum was determined using 50 mM Tris–HCl buffer, pH 8.5, at different temperatures ranging from 20 to 70 °C and the enzyme activities at different

Table 1 Summary of the purification steps for proline aminopeptidase from *S. lavendulae* ATCC 14162

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Activity yield (%)
Crude	2,650	391.5	0.15	1	100
Ammonium sulfate fractionation (50–70%)	182	94.49	0.52	3.53	24.13
Ultra-filtration (30 kDa membrane)	33.5	32.15	0.96	6.52	8.21
Anion exchange chromatography (Q-sepharose)	2.4	2.65	1.11	7.51	0.68
Gel filtration chromatography (Sephadex G-100)	1.8	2.53	1.41	9.55	0.65

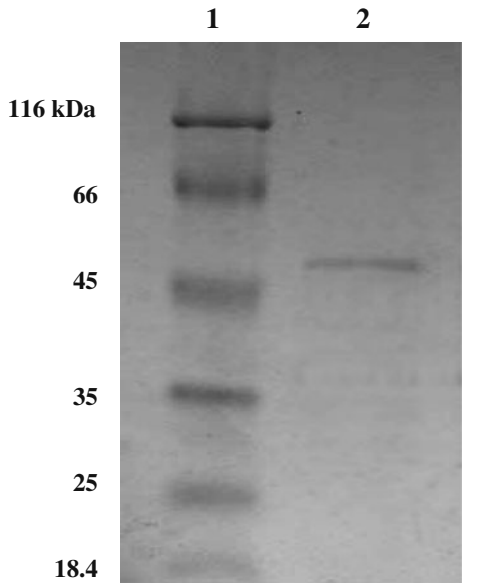

Fig. 1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified extracellular proline aminopeptidase from *S. lavendulae* ATCC 14162. Lanes: *1*, protein marker; *2*, extracellular PAP

temperatures were compared to find out the optimum temperature. The results were expressed as the percentage of the activity obtained at the optimum temperature. For the determination of thermo-stability, enzyme solutions were pre-incubated at each temperature for 10 min and followed the assay under standard conditions.

Effect of Enzyme Inhibitors and Metal Ions

The standard enzyme assay was performed in the presence of varying inhibitors. Activity was expressed as a percentage of the activity obtained in the absence of any added inhibitor. The inhibitors used are amastatin, bestatin, actinonin (1 to 10 μM), EDTA, phenanthroline (1 to 10 mM), dithiothretol, β-mercaptoethanol, and phenylmethylsulfonyl fluoride (PMSF) (1 to 10 mM). The effect of the cations on enzyme activity was determined under standard assay conditions. The metal ions used are 1 to 5 mM of $CaCl_2$, $CoCl_2$, $ZnCl_2$, $MnCl_2$, $MgCl_2$, KCl, $FeCl_2$, NaCl, $NiCl_2$, $FeCl_2$, and $CuCl_2$.

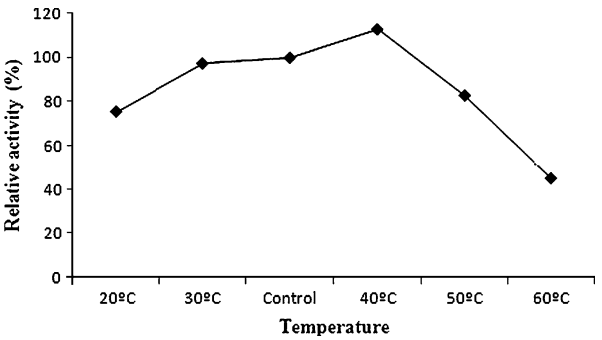

Fig. 2 Optimum temperature of proline aminopeptidase from *S. lavendulae* ATCC 14162

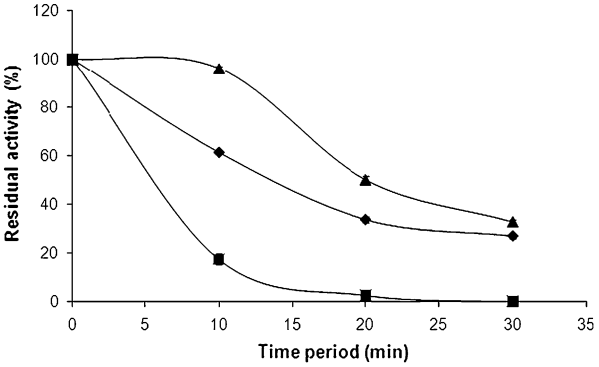

Fig. 3 Thermo-stability of proline aminopeptidase from *S. lavendulae* ATCC 14162 at 60 °C (*black square*), 50 °C (*black diamond suit*), and 40 °C (*black up-pointing triangle*) over a period of 30 min

Substrate Specificity and Kinetic Studies

The affinity of the purified aminopeptidase for various amino acid residues was determined as described previously by employing Pro-*p*NA, Leu-*p*NA, Met-*p*NA, Arg-*p*NA, Gly – *p*NA, and Val-*p*NA (Sigma) as standard substrates. The kinetic parameters of the purified enzyme were estimated for Pro-*p*NA using concentrations ranging from 0.3 to 10 mM. The kinetic parameters such as K_m and V_{max} were calculated from Lineweaver-Burk plots.

Results and Discussion

Among the 14 screened strains of *Streptomyces* species are such as *Streptomyces platensis* NRRL 2364, *Streptomyces gedanensis* IFO 13426, *Streptomyces fulvoviridis* NCIB 9804, *Streptomyces paucisporogene* ATCC 12596, *Streptomyces viridiosporous* T7 4 (=ATCC 39115), *Streptomyces aureofaciens* ATCC 10762, *Streptomyces badius* 252 (=ATCC 39117), *Streptomyces flavogriseus* IFO 13040, *S. lavendulae* ATCC 14162. Among the screened strains, *S. lavendulae* ATCC 14162 showed extracellular proline aminopeptidase activity when grown in YEME medium and it showed a maximum activity of 1.2 U/mL at 120 h of incubation at 30 °C. A summary of the purification steps is shown in Table 1. As a result of the four-step purification, nearly tenfold increase in specific activity was obtained. After the gel exclusion chromatography, the protein sample gave a prominent single band at about 60 kDa on 12% SDS-PAGE (Fig. 1). The specificity of the band was confirmed by

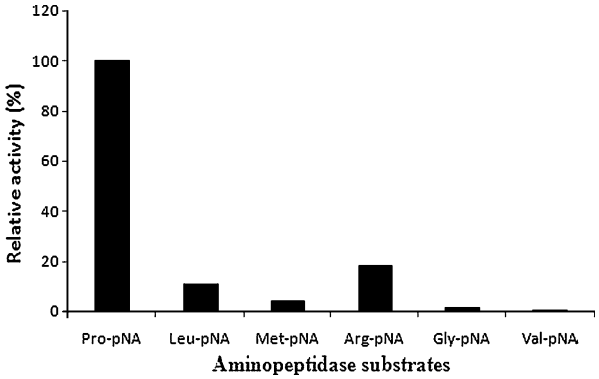

Fig. 4 Substrate specificity of proline aminopeptidase from *S. lavendulae* ATCC 14162 using Pro-*p*NA, Leu-*p*NA, Met-*p*NA, Arg-*p*NA, Gly-*p*NA, and Val-*p*NA

Table 2 Effect of chemical reagents on the activity of the extracellular proline aminopeptidase from *S. lavendulae* ATCC 14162

Chemical agent	Concentration (mM)	Relative activity (%)[a]
Control	None	100
Amastatin	0.001	99
Bestatin	0.001	100
Actinonin	0.001	166
EDTA	1	218
	10	217
1,10-Phenanthroline	1	193
	10	127
Dithiothreitol	1	93
	10	103
2-Mercaptoethanol	1	231
	10	215
PMSF	1	82
	5	64
	10	35

EDTA ethylenediaminetetraacetic acid, *PMSF* phenylmethylsulphonyl fluoride

[a] Control taken as 100%

the appearance of a clear pink band on the gel when activity staining was performed. The molecular masses of other PAPs in the denatured state were 55 to 56 kDa [15–17].

The enzyme was active over a very wide pH range, from 5.0 to 10.5. The activity was maximum (7.58 U/mL) at pH 6.5. The optimum temperature was at 40 °C as it is shown in Fig. 2. Temperature stability studies showed that, after 10 min of pre-incubation, the activity was 5.28 IU/mL at 40 °C and is further reduced to 3.88 IU/mL at 50 °C. At 60 °C, after 20 min of incubation, the enzyme lost its activity (Fig. 3). Most of the purified PAPs have an optimal pH of between 7 and 8 [15, 18–21]. The optimum temperature of the majority of PAPs is between 37 and 55 °C [19, 20]. The PAP of *Penicillium camemberti* has an optimal activity at 45 °C, as is the case for the PAP of *Debaryomyces hansenii* [18]. The study showed that the PAP from *S. lavendulae* had the optimum pH and temperature within the reported range. The substrate specificity of the purified enzyme was tested with several *p*NA derivatives of amino acids. The relative rates of hydrolysis of various substrates are

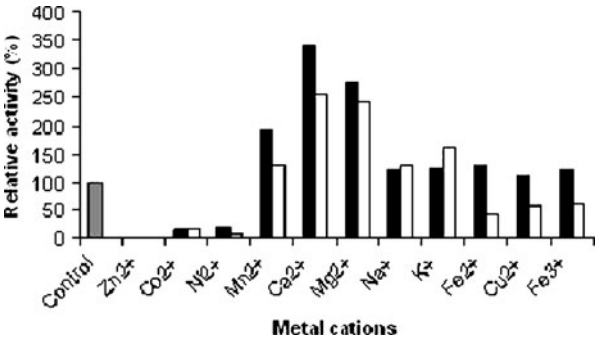

Fig. 5 Effect of selected cations at 1 mmol/L (*black square*) and 5 mmol/L (*white square*) on the enzyme activity of proline aminopeptidase from *S. lavendulae* ATCC 14162

shown in (Fig. 4). The results showed that the enzyme activity was maximum with Pro-*p*NA and it was considered as 100%.

The Michaelis constant (K_m) and maximum velocity (V_{max}) values for the enzyme were calculated as 0.23 mM and 0.087 μmol/min, respectively. Enzyme activity was strongly inhibited by the serine protease inhibitor PMSF and not by reducing agents such as dithiothreitol and β-mercaptoethanol. The enzyme was not inhibited by the metal-chelating agent ethylenediaminetetraacetic acid (EDTA) and phenanthroline. It is interesting to note that the general aminopeptidase inhibitors such as bestatin, amastatin, and actinonin did not show any effect on enzyme activity as shown in Table 2. Among the divalent ions studied, Ca^{2+}, Mg^{2+}, and Mn^{2+} enhanced enzyme activity, whereas Zn^{2+}, Co^{2+}, and Ni^{2+} had an inhibitory effect at 1 mM (Fig. 5). The proline iminopeptidase from *A. nicotianae* 9458 is a serine enzyme inhibited by PMSF and its activity was affected also by several cations (Fe^{2+}, Sn^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Co^{2+}, and Ni^{2+}). The enzyme preferentially hydrolyzed Pro-*p*NA but showed activity also on a tripeptide containing a proline residue in the middle position (Val-Pro-Leu) and on Pro-Gly. The extracellular proline iminopeptidase from *A. nicotianae* 9458 showed an activity on Leu-Pro and on Arg-Pro-*p*NA, specific substrates for prolidase and X-prolyl dipeptidyl aminopeptidase, respectively [20]. PAP of *P. camemberti* is inhibited by thiol reagents but also exhibits inhibition by di-isopropylfluorophosphate, indicating that serine residues are important for the catalytic activity [18]. PAPs from lactic acid bacteria such as *Propionibacterium shermanii*, *Lactobacillus delbrueckii*, *Arthrobacter nicotianae*, and *Hafnia alvei* are considered as serine proteases on the basis of 3,4-dichloroisocoumarin inhibition [19–22]. The activities of these enzymes were practically unaffected by EDTA, phenanthroline, and dithiotheritol but strongly inhibited by PMSF and hence they are considered to be serine peptidases. Since the PAP of *S. levenduale* is inhibited by PMSF and is not affected by other aminopeptidase inhibitors, there could be a chance that it may also come under the serine proteases.

Conclusion

S. lavendulae ATCC 14162 was found to be a good producer of extracellular proline aminopeptidase. This study provided valuable biochemical data about the properties of the PAP of *S. lavendulae* that could constitute the basis for further studies focused on its genetic and functional characterization.

Acknowledgements One of the authors (AN) is deeply indebted to Kerala State Council for Science Technology and Environment (KSCSTE) for the award of Research Fellowship for doctoral studies. The authors are also thankful to a grant from CSIR Network project (NWP 006).

References

1. Gonzales, T., & Robert-Baudouy, J. (1996). Bacterial aminopeptidases: properties and functions. *FEMS Microbiology Reviews, 18*, 319–344.
2. Chandu, D., & Nandi, D. (2003). PepN is the major aminopeptidase in *Escherichia coli*: insights on substrate specificity and role during sodium-salicylate-induced stress. *Microbiology, 149*, 3437–3447.
3. Arima, J., Uesugi, Y., Iwabuchi, M., & Hatanaka, T. (2008). *Streptomyces* aminopeptidase P: biochemical characterization and insight into the roles of its N-terminal domain. *Protein Engineering, Design & Selection, 21*, 45–53.

4. McDonnell, M., Bouchier, P., Fitzgerald, R. J., & O'Quinn, G. (1999). Purification and characterization of a lysine *p*-nitroanilide hydrolase, a broad specificity aminopeptidase, from the cytoplasm of *Lactococcus lactis* subsp. *cremoris* AM2. *The Journal of Dairy Research, 66*, 257–270.
5. Arima, J., Uesugi, Y., Iwabuchi, M., & Hatanaka, T. (2006). Dipeptide synthesis by an aminopeptidase from *Streptomyces septatus* TH-2 and its application to synthesis of biologically active peptides. *Applied and Environmental Microbiology, 72*, 4225–4231.
6. Murai, A., Tsujimoto, Y., Matsui, H., & Watanabe, K. (2004). An *Aneurinibacillus* sp. strain AM-1 produces a proline specific aminopeptidase useful for collagen degradation. *Journal of Applied Microbiology, 96*, 810–818.
7. Graham, S. C., Lee, M., Freeman, H. C., & Guss, J. M. (2003). An orthorhombic form of *Escherichia coli* aminopeptidase P at 2.4 °A resolution. *Acta Crystallographica D, 59*, 897–902.
8. Jao, S. C., Huang, L. F., Hwang, S. M., & Li, W. S. (2006). Tyrosine 387 and arginine 404 are critical in the hydrolytic mechanism of *Escherichia coli* aminopeptidase P. *Biochemistry, 45*, 1547–1553.
9. Nagy, V., Nampoothiri, K. M., Pandey, A., Rahulan, R., & Szakacs, G. (2007). Production of L-leucine aminopeptidase by selected *Streptomyces* isolates. *Journal of Applied Microbiology, 104*, 380–387.
10. Arima, J., Uesugi, Y., Iwabuchi, M., & Hatanaka, T. (2006). Study on peptide hydrolysis by aminopeptidases from *Streptomyces griseus*, *Streptomyces septatus* and *Aeromonas proteolytica*. *Applied Microbiology and Biotechnology, 70*, 541–547.
11. Arima, J., Iwabuchi, M., & Hatanaka, T. (2004). Gene cloning and overproduction of an aminopeptidase from *Streptomyces septatus* TH-2, and comparison with calcium activated enzyme from *Streptomyces griseus*. *Biochemical and Biophysical Research Communications, 317*, 531–538.
12. Tan, P. S. T., & Konings, W. N. (1990). Purification and characterization of an aminopeptidase from *Lactococcus lactis* subsp. *cremoris* Wg2. *Applied and Environmental Microbiology, 56*, 526–532.
13. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.
14. Bozic, N., & Vujcic, Z. (2005). Detection and quantification of leucyl aminopeptidase after native electrophoresis using leucine-*p*-nitroanilide. *Electrophoresis, 26*, 2476–2480.
15. Khilji, M. A., & Bailey, G. S. (1978). The purification of a bovine kidney enzyme which cleaves melanocyte-stimulating hormone-release inhibiting factor. *Biochimica et Biophysica Acta, 527*, 282–288.
16. Matsushima, M., Takahashi, T., Ichinose, M., Miki, K., Kurokawa, K., & Takahashi, K. (1991). Prolyl aminopeptidases from pig intestinal mucosa and human liver: purification, characterization and possible identity with leucyl aminopeptidase. *Biomedical Research, 12*, 323–333.
17. Ninomiya, K., Kawatani, K., Tanaka, S., Kawata, S., & Makusumi, S. (1982). Purification and properties of a proline iminopeptidase from apricot seeds. *Journal of Biochemistry, 92*, 413–421.
18. Fuke, Y., & Matsuoka, H. (1993). The purification and characterization of prolyl aminopeptidase from *Penicillium camemberti*. *Journal of Dairy Science, 76*, 2478–2484.
19. Kitazono, A., Kitano, A., Kabashima, T., Ito, K., & Yoshimoto, T. (1996). Prolyl aminopeptidase is also present in Enterobacteriaceae: cloning and sequencing of the *Hafnia alvei* enzyme-gene and characterization of the expressed enzyme. *Journal of Biochemistry, 119*, 468–474.
20. Smacchi, E., Gobbetti, M., Lanciatti, R., & Fox, P. F. (1999). Purification and characterization of an extracellular proline iminopeptidase from *Arthrobacter nicotianae* 9458. *FEMS Microbiology Letters, 178*, 191–197.
21. Gilbert, C., Atlan, D., Blanc, B., & Portalier. (1994). Proline iminopeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397: purification and characterization. *Microbiology, 140*, 537–542.
22. Panon, G. (1990). Purification and characterization of a proline iminopeptidase from *Propionibacterium shermanii* 13673. *Le Lait, 70*, 439–452.