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*Full Length Research Paper*

# Specific aminopeptidases of indigenous *Lactobacillus brevis* and *Lactobacillus plantarum*

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**Lactic acid bacteria play an important role in milk coagulation and cheese ripening. To select strains showing interesting industrial features, two indigenous lactobacilli (*Lactobacillus brevis* and *Lactobacillus plantarum*) were studied for aminopeptidase activity. Cell and cells free extract were tested for leucyl aminopeptidase activity on the chromogenic leucyl-p-nitroanilide substrate. Intracellular and membrane enzymes were solubilized with glycine /lyzozyme treatment then purified by ammonium sulphate precipitation followed by Sephadex G100 and diethylaminoethyl (DEAE) ions exchange chromatography's separation. The molecular weight of denatured proteins was estimated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Effects of several parameters, pH, temperature, some ions and inhibitors on purified enzyme activity were studied. Cellular aminopeptidase activity was higher for CHTD 27 strain than BH14 strain. No aminopeptidase activity was noted in the cell free extract. The results of chromatography sephadex G100 combined to those of electrophoresis allowed suggesting a dimer structure for the native enzyme. The *Lb* CHTD27 purified enzyme showed maximal activity at pH 6.6 and at 40°C. This enzyme was partially inhibited by ethylenediamine acetic acid (EDTA) and $Cu^{2+}$ ions but increased by $Na^{2+}$ and $Co^{2+}$ ions. The aminopeptidase extracted from *Lb* BH14 was inhibited by EDTA and phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride (PMSF), its maximal activity was observed at pH 7.5 and 40°C. In addition to other characteristics as proteolysis and autolysis, in this paper we showed that both studied strains were also able to degrade peptide with specific peptidases which are important characters in cheese manufacturing.**

**Key words:** Camel milk, *Lactobacillus*, proteolysis, leucyl aminopeptidase activity.

## INTRODUCTION

Cheese manufacturing includes physical and chemical changes in product followed by flavour characteristics development. These changes are principally attributed to lactic acid bacteria proteolysis, lipolysis and lactose fermentation (Juillart et al., 1996; Mc Sweeney and Sousa, 2000; Børsting and Ardö, 2011). Through their complex enzymatic equipment (proteases, peptidases etc), lactobacilli play an important role in cheese ripening (Khalid and Marth, 1990; Masuda et al., 2005). Among these enzymes, various peptidases hydrolyse peptides into amino acids required for optimal bacterial growth in milk and used as aroma compound precursors (Mierau et al., 1996; Christensen and Steel, 2003; López, 2008). Some lactobacilli enzymes are extracellular but majority are liberated after bacterial cells autolysis (Lortal et al., 1989, 1991; Chapot-Chartier, 1996).

The industrial interest of lactic acid bacteria and their use in health have been the subject of several studies on a large scale, the evolution of knowledge led to the selection and development of new strains. In this way LBMB (Laboratoire de Biologie des Microorganismes et Biotechnologie de l'Université d'Oran ALGERIE) set the

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goal of preparing a collection of indigenous interesting lactic acid bacteria strains (Karam, 1995).

In order to select lactobacilli which showed technological traits, in this study we studied leucyl aminopeptidase activity of *Lactobacillus brevis* CHTD27 and *Lactobacillus plantarum* BH14 isolated from camel milk. This type of enzyme contributes to bitterness reduction (Thivierge, 1999; Tchorbanov et al., 2011), so strains which present this activity are in demand.

## MATERIALS AND METHODS

### Bacterial strains and culture conditions

*L. brevis* CHTD27 and *L. plantarum* BH14 used in this study were originally isolated from raw camel milk collected in *Tindouf* and *Timmimoun* areas respectively for CHTD27 and BH14. They were previously selected as proteolytic and autolytic strains (Roudj et al., 2009). Bacterial growth was prepared in MRS broth (Man et al., 1963) at 30°C for 24 h.

### Enzymes assay

Aminopeptidase (AP) activity was tested by using chromogen leucyl paranitroanilide substrate prepared at 20 mM in absolute methanol according to the method of El Soda and Desmazeaud (1982). Reaction mixture contained 200 µl potassium phosphate buffer (0.1M pH7.0), 25 µl of substrate (freshly prepared) and 50 µl of enzyme. The mixture was incubated at 37°C for 24 h. The reaction was stopped by adding 300 µl of acetic acid solution (10%). After centrifugation (12000 rpm/10 min), supernatant absorbance was read against control sample prepared and incubated similarly without enzyme. Hydrolysis of substrate and nitroanilide liberation was determined by measuring absorbance at 410 nm. The supernatant of liquid culture (MRS/2% milk) and cells were tested for AP activity as described above.

### Effects of pH and temperature on cell aminopeptidase activity

Effects of pH and temperature on cell AP activity were determined to carry all steps of partial purification of aminopeptidase at both optimum pH and optimum temperature. Cell cultures were incubated at 6, 20, 30, 40, 50 and 60°C. For pH effect, medium was prepared by potassium phosphate buffer 0.1 M at pH 5, 6, 6.1, 6.9, 7.3 or 8.3.

### Extraction, purification and characterization of enzyme

#### *Preparation of cell free extract*

Enzymes were extracted from cells by both actions of glycine and lysozyme. Cells culture was prepared on MRS medium supplemented with 4% glycine. After 18 h of incubation at 37°C optical density ($OD_{640nm}$ = 1), cells were collected by centrifugation (12000 rpm/10 min/4°C). The pellet was washed twice with potassium phosphate buffer (0.1 M pH7.0) and suspended in the same buffer supplemented with 10 mM of lysozyme. After 2 h of lysozyme treatment at 37°C, the mixture was centrifuged (12000 rpm/10 min/4°C). The resulting supernatant was designed as the cell free extract.

#### *Partial purification of aminopeptidases*

For both strains the cell free crude was fractioned by salting out using two concentrations 50% then 80% of solid ammonium sulfate (Goldsteine *et al*., 2002).

The ammonium sulfate protein fraction was dissolved in 0.1 M potassium phosphate buffer pH 7.0 and dialysed in first against distilled water then against sucrose. The 3 times concentrated fraction was applied to Sephadex G100 (Pharmacia) column (20 × 1.2 cm) equilibrated with Tris-HCl 0.05 M pH 7.0 buffer. Proteins were eluted by the same buffer, and fractions of 1.5 ml were collected. The active fraction obtained after the first chromategraphy was loaded on to a DEAE cellulose (D52 Whatman) column prepared as described by Deneuville (1991). Enzymes were eluted by discontinuous gradient of NaCl (1% then 3%) in potassium phosphate buffer (0.1 M pH 7.0). Fractions of 2 ml were recuperated.

Proteins concentrations were determined by the method of Bradford (1976) using BSA as standard. The molecular weights of the denatured enzymes were estimated by SDS gel electrophoresis (SDS PAGE) using (5%) stacking gel and (13%) resolving gel and weight markers (Catalase 250 kDa, BSA 67 kDa, Olvabumin 43 kDa, Pepsin A 35 kDa, Chymotrypsin 25 kDa) prepared according to the method of Laemmli (1970).

#### *Effect of pH and temperature on enzyme activity*

The effect of temperature on aminopeptidase activity was determined after 15 min incubation of enzyme at 30, 40 or 50°C prior to add the substrate then the mixtures was incubated for 24 h. The effect of pH was studied by using potassium phosphate buffer 0.1 M at pH 5.1, 5.6, 6.6, 7.5 or 8.0. The enzyme was previously incubited 15 min in each buffer before adding substrate. Incubation was extended for 24 h at 40°C.

#### *Effect of inhibitors agents and metal ions on enzyme activity*

Effects of chemicals agents phenylmethylsulfonylfluoride (PMSF), β-mercaptoethanol, EDTA (5 mM then 10 mM ), and the following di-ionic agents $CoCl_2$, $CaCl_2$, $KCl$, $ZnCl_2$, $NaCl$, $MnCl_2$, $MgCl_2$ , $CuCl_2$ (5 mM then 10 mM) were determined. Chemicals inhibitors and ions were added to the reaction (potassium phosphate buffer 0.1 M pH 6.9 + enzymes). Mixture was incubated for 15 min at 40°C before adding substrate then the incubation was extended for 24 h in the same temperature.

In every case, enzyme activity was expressed as the percentage of activity obtained with or without the studied compound. Every experience was repeated many times (more than 3 tubes were prepared during the OD measure) for the result confirmation.

## RESULTS

### Aminopeptidase activity

The two studied strains produced aminopeptidases, it was revealed by hydrolysis of the chromogen substrate which becomes yellow (measured at 410 nm) after liberation of nitroanilide.

This activity was important for cells of *L. brevis* CHTD27 ($OD_{410nm}$ = 2.85) then the cells of *L. plantarum* BH14 ($OD_{410nm}$ = 1.96). A negligible AP activity was noted in the extracellular supernatant of liquid culture, $OD_{410nm}$ =

**Table 1.** Results of purification steps.

| Fraction | Protein (mg) | Cat act (nanokat) | Sp act (nanokat/mg) | P | R (%) |
|---|---|---|---|---|---|
| **CHTD27** | | | | | |
| CFE | 1340 | 193.52 | 0.144 | 1.00 | 100 |
| $SA_{80}$ | 294.83 | 159.21 | 0.54 | 3.75 | 82.27 |
| G100 | 65 | 70.69 | 1.09 | 7.55 | 36.52 |
| DEAE | 38 | 45.98 | 1.21 | 8.40 | 23.76 |
| **BH14** | | | | | |
| CFE | 980.00 | 134.50 | 0.14 | 1.00 | 100 |
| $SA_{80}$ | 263.52 | 131.76 | 0.50 | 3.57 | 97.96 |
| G100 | 80.0 | 56.27 | 0.70 | 5.02 | 29.10 |
| DEAE | 12.38 | 8.92 | 0.72 | 5.14 | 6.63 |

**CFE:** crude free extract; **$SA_{80}$:** 80% ammonium sulphate saturation; **G100:** Sephadex G100 chromatography; **DEAE:** DEAE cellulose chromatography; **Cat act**: catalytic activity; **Sp act**: specific activity; **P**: purity; **R**: yield.

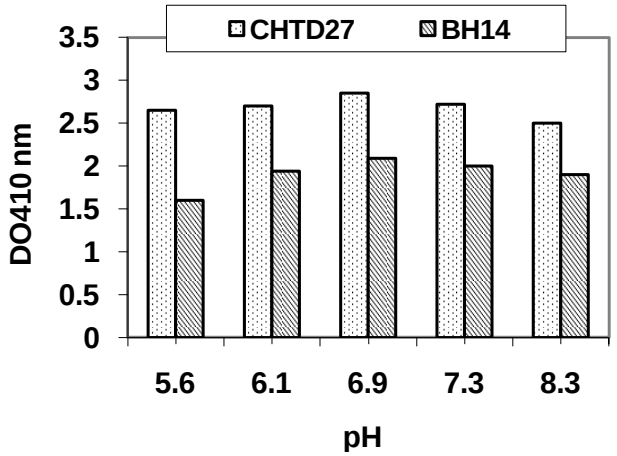


**Figure 1.** Effect of pH on cells AP activities.

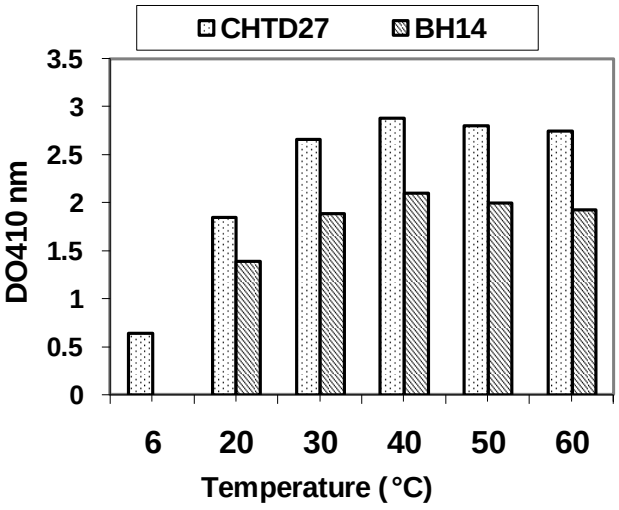


**Figure 2.** Effect of temperature on cells AP activities.

0.0815 for *L. brevis* CHTD27 and $OD_{410nm}$ = 0.0915 for *L. plantarum* BH14.

## Optimal conditions of cells aminopeptidase activities

Temperature and pH conditions were examined in order to have maximal cells AP activities for the remaining work. According to the results (Figures 1 and 2), maximal activities were obtained for both strains at temperature of 40°C and at pH 6.9. These two parameters were retained for the following steps.

## Subcellular localisation of aminopeptidase

### *Enzyme extraction*

Various procedures were used for cells aminopeptidase extraction (toluene, potassium phosphate buffer 0.1 M pH 7.2, NaCl 0.9%). These methods proved to be inefficient and they trained interference problems with chromogen substrate during measuring. The method assembling culture in the presence of glycine and lysozyme treatment allowed the extraction of these enzymes, effectively, catalytic activities reached 0.144 nanokatals/mg for L. brevis CHTD27 and about 0.1 nanokatals/mg for L. plantarum BH14 as measured in brute extracts (Table 1).

## Enzymes purification from extract

The specific peptidase activity observed with Leu-pNA as substrate at each purification step is presented in Table 1. The enzyme was purified about 9-fold from the CHTD27 cell-free extract and 5 fold from the BH14 cell-free extract. Chromatograms are shown in Figures 3 and 4.

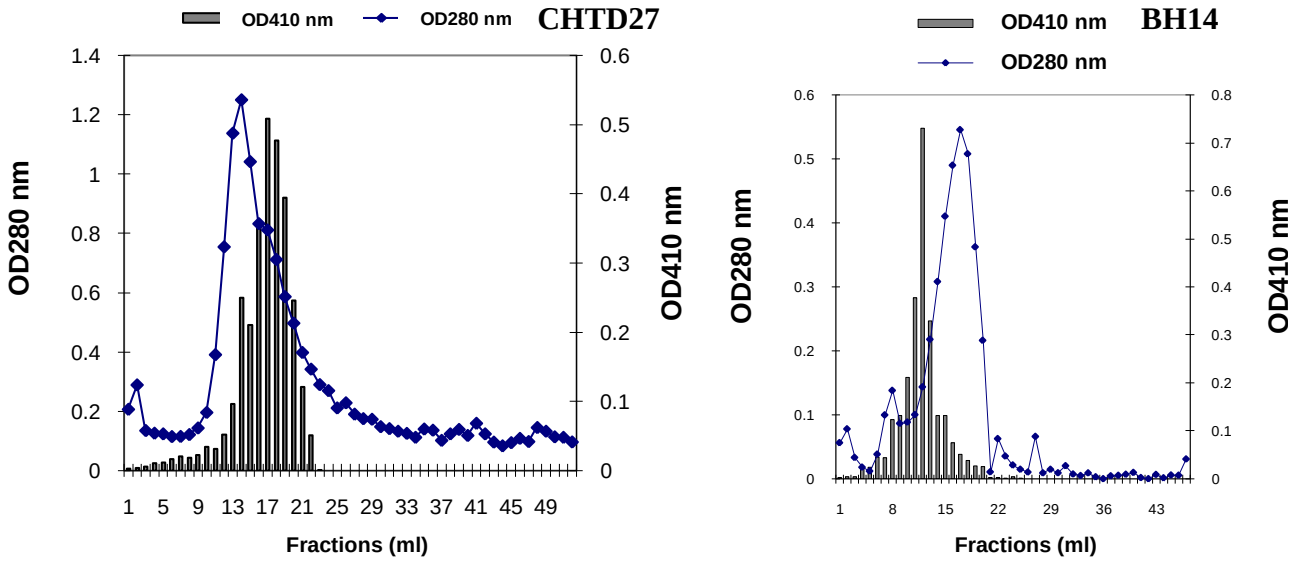


**Figure 3.** Séphadex G100 chromatograms of $SA_{80}$ fraction. Absorbance of protein contents was measured at 280 nm and of AP activity at 410 nm.

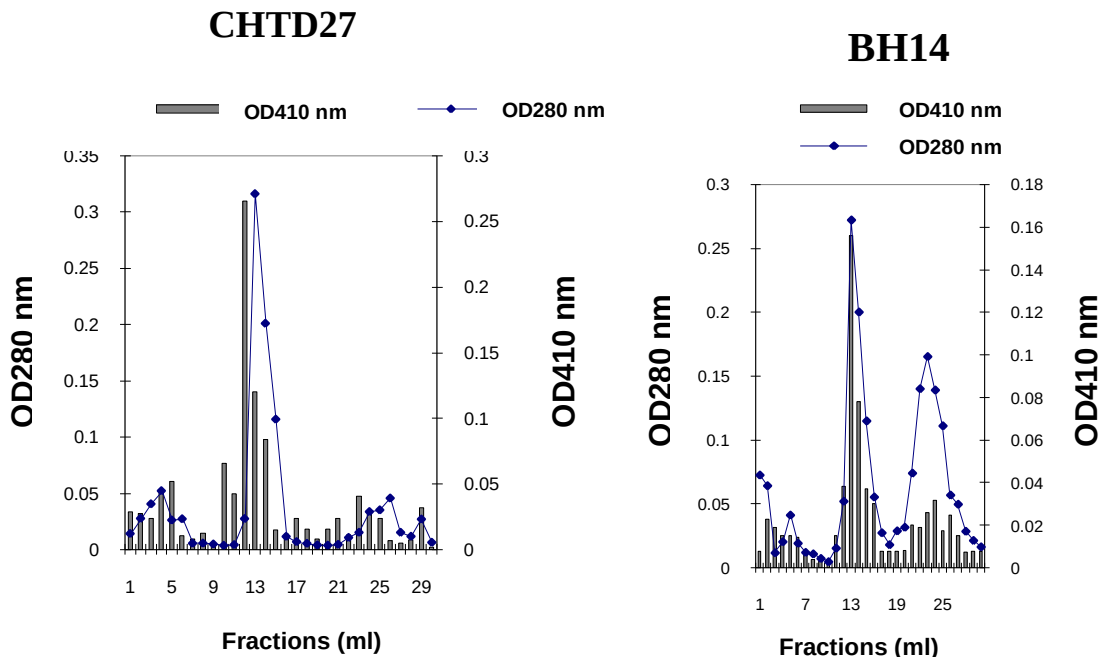


**Figure 4.** DEAE Cellulose Chromatograms of G100 fractions. Absorbance of protein contents was measured at 280 nm and of AP activity at 410 nm.

## Electrophoretical analysis of the purified enzymes

The molecular weight of the purified enzyme as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was about 43 kDa (Figure 5). The molecular mass of the native enzyme was previously estimated to be close to 100 kDa by Sephadex G100 column filtration and hence both results allowed suggesting that the native enzyme is a dimeric molecule.

## Biochemical characteristics of the purified enzymes

Effects of several parameters, pH, temperature, ions and inhibitors on enzyme activity were evaluated and results

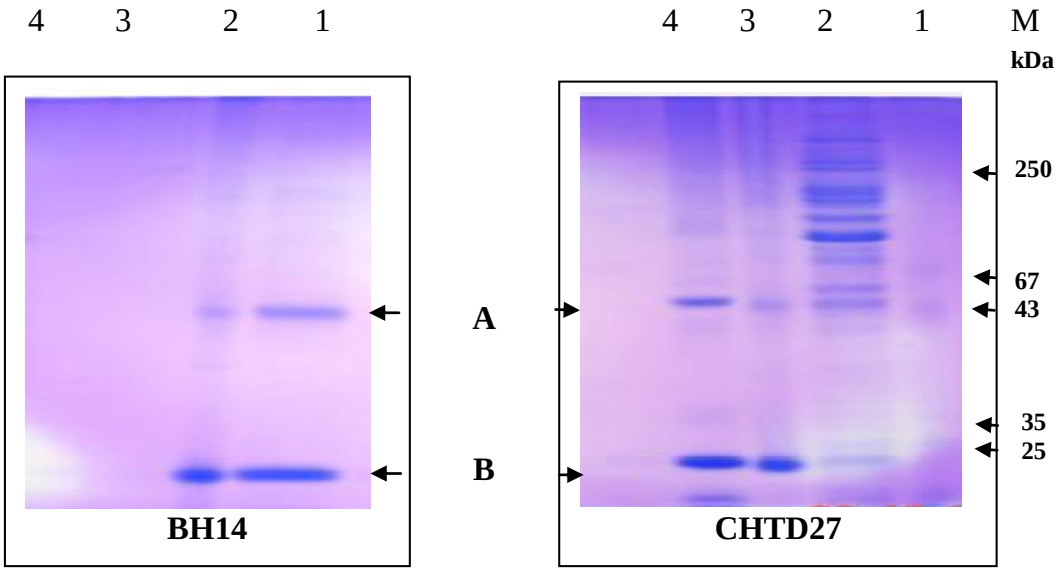


**Figure 5.** Electrophoresis of the different enzymatic fractions. Lanes: 1: Crude extract; 2: Ammonium sulphate precipitation fraction; 3: Sephadex G100 fraction; 4: DEAE fraction; M: MW markers. Arrows indicate bands of: A: Aminopeptidase; B: Lyzozyme.

**Table 2.** Effects of several parameters on AP activity.

| Stains parameter | CHTD27 (%) | | BH14 (%) | |
|---|---|---|---|---|
| **Temperature (°C)** | | | | |
| 30 | 87.5 | | 85 | |
| 40 | 100 | | 100 | |
| 50 | 46 | | 90 | |
| **pH** | | | | |
| 5.0 | 27.6 | | 58.7 | |
| 5.6 | 34.6 | | 79.5 | |
| 6.6 | 100 | | 85.4 | |
| 7.5 | 67.8 | | 100 | |
| 8.0 | 40.5 | | 73.3 | |
| **Inhibitor** | **5 mM** | **10 mM** | **5 mM** | **10 mM** |
| PMSF | 94.42 | 64.6 | 92.44 | 25.7 |
| β —mercaptoethanol | 98.07 | 92.3 | 166.6 | 98.94 |
| EDTA | 76.9 | 76.9 | 47 | 35 |
| **Ions** | | | | |
| $CaCl_2$ | 77.35 | 89.14 | 76.68 | 96 |
| KCl | 76.71 | 87.42 | 100 | 100 |
| $ZnCl_2$ | 61.84 | 64.24 | 98.94 | 98.9 |
| NaCl | 68.8 | 141 | 92.9 | 101.1 |
| $MnCl_2$ | 77.51 | 106.2 | 51.4 | 100 |
| $MgCl_2$ | 66.08 | 74.5 | 101.1 | 100 |
| $CuCl_2$ | 54.5 | 28.2 | 30.5 | 27.6 |
| $CoCl_2$ | 77.1 | 130 | 102.3 | 101.1 |

The percentage represents the residual activity.

are summarized in Table 2. The enzyme extracted from BH14 strain was activated by β mercaptoethanol at 10 mM (166.6%) and strongly inhibited by EDTA (35%) and PMSF at 10 mM (25.7%) while the enzyme extracted from CHTD27 strain was partially inhibited by EDTA and PMSF; however no effect of β mercaptoéthanol was observed on this enzyme. For both strains the AP activity was strongly inhibited by $Cu^{+2}$ (54.5%, 28.2% for CHTD27 and 27.6%, 30.5% for BH14) respectively at 5 and 10 mM. The maximum of the enzymatic activity was noted at pH 6.6 and 40°C for CHTD27 and pH 7.5 and 40°C for BH14.

## DISCUSSION

The two studied strains express variation in cell aminopeptidases activity which was also reported by Thivierge (1999) for mesophilics *Lactococcus* strains and Liu et al. (2010) for lactobacilli strain. The absence of aminopeptidase activity in extracellular fraction allowed us to conclude that the studied enzyme is not or little exuded in the extracellular medium by our strains. Membrane and cytoplasm localisations were usually indicated for lactobacilli pepN (Eggimann and Bachmann, 1980; Atlan et al., 1989, 1990; Cholette and Mc Kellar, 1990; Tsakalidou et al., 1993; Choi et al., 1995; Macedo et al., 2003; Pan and Tanokura, 2004). These results are evident because such enzyme plays an important role in cellular nutrition by supplying requisites amino acids for cells growth. The extracellular liberation of such enzymes leads cells to not use milk caseins, so it is not the case for lactic acid bacteria and notably for lactobacilli which use the caseins in milk as an additional source of nitrogen (Monnet et al., 1993; Pritchard and Coolbear, 1993).

The two strains synthesized aminopeptidases during logarithmic stage of growth between 6 and 18 h of culture. This activity was stable during stationary phase of growth. Reduction in production of AP was noted with cellular density diminution (after 20 h of culture). This cells autolysis was not accompanied by AP activity augmentation (Roudj et al., 2009). This may indicate a membrane localisation for enzymes of both strains.

To extract enzymes we prepared the culture in the presence of glycine then cells were kept under lysozyme action. Several techniques for extracting AP from cells have been cited in the literature such as osmotic shock (Sanz and Toldra, 2002; Tsakalidou et al., 1993; Monnet et al., 1995; Tobiassen et al., 1997; Magboul and Sweeney, 1999a, Sanz and Toldra, 2002;), lysozyme and ultrasonic treatments or cell French Pressure (Eggimann and Bachmann, 1980; Miyakawa et al., 1992) or glass beads Beater (Bolumar *et al*., 2003).

Enzyme extraction by glycine/lysozyme treatment supports membrane AP localisation hypothesis for both studied strains. When cultured with high concentration of glycine, growing cells incorporate this molecule into their wall, which replace alanine at different positions in peptidoglycan, so wall is fragilized and protease are easily liberated (Hammes et al., 1973). The lysozyme treatment destroys membrane o-sides links.

The purified enzymes showed a dimeric structure concluded from electrophoresis and gel filtration chromatography results. This result is similar to the result of other authors obtained for peptidase of *L. curvatus* DPC2024 and *L. plantarum* ESB5004 (Magboul and Sweeney, 1999a; Macedo et al., 2003). However, the dimeric structure differs from work which spoke about monomeric or trimeric *Lactobacillus* peptidases (Eggimann and Bachmann, 1980; Miyakawa et al., 1992; Tsakalidou et al., 1993; Gobbetti et al., 1996; Pan and Tanokura, 2004). Results obtained for *L. plantarum* BH14 and *L. brevis* CHTD27 and those of other authors for lactobacilli strains demonstrate an important diversity of AP in *Lactobacillus* group (El soda et al., 1983). Effectively mesophilic homofermentative lactobacilli, mainly *L. brevis*, *L. casei*, *L. paracasei*, *L. plantarum* and *L. curvatus* are the common strains of nonstarter culture incoporated into starter microflora to control and accelerate cheese ripening by numerous peptidases (Tobiassen et al., 1997). These peptidases are encoded in all lactic acid bacteria genomes usually with one gene per genome. For instance, aminopeptidases PepC, PepN, and PepM, and proline peptidases PepX and PepQ are present in all genomes, some lactic acid bacteria genomes have two peptidase homologs, possibly with the same function (Liu et al., 2010). Other essential peptidases such as endopeptidase PepO and dipeptidase PepV are encoded by multiple paralogous genes (Liu et al., 2010).

Aminopeptidase of *L. brevis* CHTD27 has characteristics usually observed in aminopeptidases of other *Lactobacillus* strains. It is like to be a metallo protein activated by $Co^{2+}$ and inhibited by $Cu^{2+}$. The optimum pH and temperature values for maximal enzyme activity were respectively 6.9 and 40°C (Eggimann and Bachmann, 1980; Gobbetti et al., 1996; Tsakalidou et al., 1993; Sanz and Toldra, 2002; Nandan et al., 2010).

Aminopeptidase extracted from *L. plantarum* BH14 is different; it is a metallo enzyme with serine in its catalytic setting. The enzyme activity was inhibited by $Cu^{2+}$ ions and the maximum of this activity was observed at pH 7.5 and 40°C. Some *Lactobacillus* aminopeptidases have also thiol or cysteine groups in their catalytic sites (Miyakawa et al., 1992; Magboul and Mc Sweeney, 1999b). *L. plantarum* BH14 aminopeptidase activity was increased by β mercaptoethanol. This rise was explained by the pH conditions of the reactive mixture according to Cholette and Mc Kellar (1990). According to literature, gene analysis suggests that some peptidases genes in *Lactobacillus* strains share the same neighbor genes, except for *L. plantarum* and both for *L. brevis* (El soda et al.,

1983, Liu et al., 2010). For example the *L. plantarum* pepM gene (LPL_28377183) is flanked by a methionine metabolism related operon (cysK_cblB/cglB_cysE). Therefore, the pepM gene in *L. plantarum* may have a broader function, probably utilizing proteins and peptides as methionine pool, in addition to the classic PepM function for N-terminal maturation of proteins (Liu et al., 2010).

## Conclusion

Our investigation contributed to acquire more knowledge about properties of enzymes by study proteolysis of two indigenous *Lactobacilli* isolated from raw Algerian camel milk to select new strains with interesting technological traits requisite in cheese manufacturing. The two strains have extracellular proteolytic and high autolytic activities (Roudj et al., 2009), desirables characteristics in milk coagulation and cheese ripening (El Soda et al., 2000; Madkor et al., 2000; Liu et al., 2010; El-Tanboly et al., 2010). In this paper we proved that these strains are able to hydrolyse leucyl paranitroanilide by particular amino-peptidase which is also an important character to avoid bitterness during milk coagulation. For their proteolytic and autolytic activities the two strains can be used as starter culture necessary in the early stages of cheese production or as nonstarter culture which contribute to flavor development in some varieties of cheeses. These strains can be used in culture alone or with other types of lactic acid bacteria *Leuconostoc*, *Lactococcus*, or with others microorganisms as yeast or fungal in mixed culture (closed mixed).

The aminopeptidase activity was slightly important for cells of *L. brevis* CHTD27 then *L. plantarum* BH14. The enzymes implicated in this activity are metalloenzymes with maximal activity at 40°C for both strains. Aminopeptidases of *Lactobacillus brevis CHTD27* presented properties usually noted in N aminopeptidases isolated from Lactobacilli, the one obtained from *Lactobacillus plantarum BH14* showed particular characteristics observed in some *Lactobacillus.* Our results point to that these enzymes are composed of two subunits with molecular weight of 43 kDa which is a result already watched for some *Lactobacillus* aminopeptidases. In further work we need to test these peptidases on the caseinolytic products especially when some *Lactobacillus* strains have positive effect on cheese flavour (*L plantarum*, *L casei*) and others are responsible for bitterness development like *L. brevis* (Khalid and Marth, 1990; Tobiassen et al., 1997; Tchorbanov et al., 2011).

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