Purification and Characterization of a Dipeptidase from *Lactobacillus casei* ssp. *casei* IFPL 731 Isolated from Goat Cheese Made from Raw Milk

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ABSTRACT

A dipeptidase was purified to homogeneity from the cell-free extract of *Lactobacillus casei* ssp. *casei* IFPL 731 by a combination of heat treatment, hydrophobic interaction chromatography, anion-exchange chromatography, and gel filtration. A purification factor of 395-fold was obtained, and yield was 20%. The dipeptidase was shown to be a metal-dependent enzyme; optimal activity was at pH 7.5 and 60 to 75°C, and the enzyme had a high degree of thermal stability. Molecular mass was estimated by SDS-PAGE and gel filtration to be 46 kDa, which suggested that the enzyme existed as a monomer. Enzyme activity was most effectively inhibited by metal-chelating agents, reducing agents, or sulfhydryl group reagents. After inhibition with phenanthroline, activity was partially restored by Co^{2+} and Mn^{2+}. The kinetics of Phe-Ala and Leu-Leu did not follow Michaelis-Menten saturation kinetics but exhibited a mixture of positive and negative cooperativity for the successive binding of molecules of the same substrate. (**Key words**: *Lactobacillus casei*, dipeptidase, purification, characterization)

Abbreviation key: **pI** = isoelectric point.

INTRODUCTION

Although starter lactococci are usually the dominant flora during the initial weeks of cheese ripening, lactobacilli are frequently found in large numbers during the late ripening period (19). The capacity of lactobacilli to hydrolyze peptides and to degrade amino acids (16) to produce further compounds has stimulated new studies aimed at evaluating the desirable peptidases produced by this group of microorganisms.

The proteolytic system of lactococci has been extensively studied. The system consists of a proteinase that is bound to the cell wall and several probably intracellular peptidases: at least one endopeptidase, a prolidase, a dipeptidase, a tripeptidase, and several aminopeptidases of various specificities (aminopeptidase N, aminopeptidase C, aminopeptidase A, X-prolyl dipeptidyl aminopeptidase, proline iminopeptidase, and aminopeptidase P). Recently, interest in the proteolytic system of lactobacilli has increased, and similarities and differences in the proteolytic system of lactic acid bacteria are being reported (14).

Lactobacillus casei occurs in a wide variety of cheeses, mainly as adventitious flora (26). This organism has been reported to enhance cheese flavor when added to the starter culture in semi-hard goat cheese and Cheddar cheese (21, 24). Although *Lb. casei* exists in greater numbers and exhibits higher peptidase and esterase activities than do other strains (4, 17), only a few peptidases have been purified and characterized from this group: an X-prolyl dipeptidyl aminopeptidase, an aminopeptidase, and an iminopeptidase (1, 2, 7, 11). El-Soda et al. (8) separated and characterized three exopeptidases (an aminopeptidase, a dipeptidase, and a specific carboxypeptidase), but did not achieve complete purification and characterization.

This paper describes the purification and characterization of a dipeptidase from *Lb. casei* ssp. *casei* IFPL 731 (Table 1). This enzyme is different from other dipeptidases from lactic acid bacteria because of its high temperature optimum and complex kinetic behavior.

MATERIALS AND METHODS

Organism, Growth, and Preparation of Cell-Free Extract

Lactobacillus casei ssp. *casei* IFPL 731 was isolated from artisanal cheese made from caprine milk (9). The organism was subcultured twice overnight in 10 ml of MRS medium at 30°C and then grown at 30°C in 5 L of MRS (Oxoid, Unipath Ltd., Basingstocke, Hampshire, England) with an inoculum of 2%. Cells

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TABLE 1. Summary of the purification procedure for dipeptidase from *Lactobacillus casei* ssp. *casei* IFPL 731.

Purification step	Total protein	Total activity[1]	Specific activity	Purification	Activity yield
	(mg)	(units)	(units/mg)	(–fold)	(%)
Cell-free extract	198.80	119,000	598.6	1.0	100
Heat treatment	66.30	136,380	2066.4	3.4	115
Hydrophobic interaction	1.07	42,478	39,699.9	66.3	36
Anion-exchange	0.25	26,362	105,449.2	176.2	22
Gel filtration	0.10	23,632	236,320.5	394.8	20

[1]Activity is expressed in units as nanomoles of leucyl-leucine hydrolyzed per minute under the conditions assay.

were harvested by centrifugation (10,000 × *g* for 15 min at 4°C) at the late exponential growth period. Cells were washed twice and resuspended in 70 ml of 0.02 *M* Tris·HCl buffer (pH 7.0).

For disruption, the cell suspension was mixed (1:1, vol/wt) with glass beads (150 to 212 μm; Sigma Chemical Co., St. Louis, MO) and shaken in an Osterizer (Sunbeam-Oster Co., Inc.) (4 × 4 min at 4°C). Glass beads, unbroken cells, and cell debris were removed by centrifugation (15,000 × *g* for 30 min at 4°C), followed by filtration through a Whatman number 1 filter paper. The clear supernatant, which constituted the cell-free extract, was then treated with deoxyribonuclease and ribonuclease (8.0 μg/ml; Boehringer Mannheim GmbH, Mannheim, Germany) for 30 min at 20°C.

Enzyme Purification

Heat treatment. The cell-free extract was first fractionated by incubation at 60°C for 30 min, then cooled, and centrifuged at 20,000 × *g* for 30 min at 4°C. The supernatant was used for further purification. All subsequent chromatographic steps were carried out at 4°C using FPLC (Pharmacia, Uppsala, Sweden).

Hydrophobic interaction chromatography. A fractogel TSK butyl-650 (E. Merck, Darmstadt, Germany) column (19 cm × 2.6 cm i.d.) was equilibrated with 0.02 *M* Tris·HCl buffer (pH 7.0) containing 1.5 *M* $(NH_4)_2SO_4$. The supernatant obtained after heat treatment was made to 1.5 *M* by addition of solid $(NH_4)_2SO_4$ and applied to the column. After the column was washed with three column volumes of starting buffer, bound proteins were eluted in a linear decreasing gradient of $(NH_4)_2SO_4$, 1.5 to 0 *M*, at a flow rate of 5 ml/min. Fractions that had maximum dipeptidase activity were concentrated in an ultrafiltration cell (Amicon, Danvers, MA) using Amicon filter YM 10 and desalted by passing through a Sephadex G-25M column (5 cm × 1.6 cm i.d.; Pharmacia) that had been previously equilibrated with 0.02 *M* Tris·HCl buffer (pH 7.0).

Anion-exchange chromatography. The desalted fraction from hydrophobic interaction chromatography was applied to a Mono Q HR 5/5 column (Pharmacia) equilibrated with 0.02 *M* Tris·HCl buffer (pH 7.0). Noninteracting proteins, among them dipeptidase, were eluted with equilibrating buffer at a flow rate of 1 ml/min.

Gel filtration. Active fractions that were obtained after anion-exchange chromatography were concentrated by ultrafiltration and applied to a Superose 12 HR 10/30 column (Pharmacia) that had been previously equilibrated with 0.02 *M* Tris·HCl buffer (pH 7.0). Enzyme was eluted in equilibrating buffer at a flow rate of 0.3 ml/min. Fractions with the highest dipeptidase activity were stored at –90°C for further studies.

Enzyme Assay

Dipeptidase activity was determined by a coupled L-amino acid oxidase-peroxidase enzyme system (25) using Leu-Leu as substrate (Sigma Chemical Co.). The reaction mixture contained 100 μl of peroxidase (5 mg/ml in 0.8 *M* $(NH_4)_2SO_4$), 50 μl of L-amino acid oxidase (2 mg/ml), 50 μl of *o*-dianisidine (11.5 m*M*), 740 μl of 0.05 *M* Tris·HCl (pH 7.5), 50 μl of substrate (40 m*M* Leu-Leu), and 10 μl of an appropriate concentration of enzyme. Samples were incubated at 50°C for 2 to 7 min, over which time the reaction rate was linear. Substrate hydrolysis was monitored continuously in a Shimadzu UV-1601 spectrophotometer with a thermostatically CPS-240 controller (Shimadzu, Kyoto, Japan) by following the amount of oxidized *o*-dianisidine formed at 436 nm. Enzyme activities were calculated by using a molar absorbance coefficient of 8100/*M* × cm for oxidized *o*-dianisidine (25). Control assays containing no enzyme were also car-

ried out, and the rate of formation of oxidized *o*-dianisidine for the substrate was subtracted from that in the presence of enzyme. All assays were conducted in duplicate.

For characterization studies, enzyme activity was determined by measuring the release of α-amino acids by the modified cadmium-ninhydrin method that was described by Doi et al. (6). Standard conditions were 70 μl of 0.05 *M* Tris·HCl buffer (pH 7.5), 20 μl of 10 m*M* Leu-Leu, and 10 μl of purified enzyme solution. After the solution was incubated for 10 min at 50°C, 10 μl of sample solution were taken, and subsequently 90 μl of 30% acetic acid (to stop the reaction) and 900 μl of ninhydrin reagent were added. The samples were heated for 5 min at 84°C and then cooled; absorbance was measured at 507 nm.

Determination of Molecular Mass

The molecular mass of the enzyme was estimated by SDS-PAGE and by gel filtration on a Superose 12 HR 10/30 column; SDS-PAGE was performed using the Bio-Rad Mini-Protean cell (Bio-Rad, Munich, Germany) and the Laemmli buffer system (15) with gels containing 4 to 20% polyacrylamide gradient. Proteins were stained with Coomassie Blue R. The molecular mass of the purified dipeptidase was estimated by reference to migration of marker proteins of the SDS-6H standard (Sigma Chemical Co.). For determination of molecular mass by gel filtration, a Superose 12 column was calibrated using a kit for molecular mass markers for gel filtration (GF-200; Sigma Chemical Co.).

Isoelectric Focusing

Isoelectric focusing was performed on a Phast system (Pharmacia) using a 5% polyacrylamide gel containing Pharmalytes 4.0 to 6.5 (Phastgel; Pharmacia). Isoelectric conditions and staining with PhastGel Blue R followed the procedures in the instruction manual that was provided by the manufacturer. The isoelectric point (**pI**) of the enzyme was determined using the Pharmacia low pI calibration kit.

Protein Determination

Protein content was determined by the micromethod of Bradford (3) using the Bio-Rad protein assay, and bovine serum albumin (fraction V; Merck) was the standard.

Characterization of the Dipeptidase

Effect of pH and temperature. The effect of pH (at 50°C) on dipeptidase activity was examined by the cadmium ninhydrin method using acetate buffer (pH 4.0 to 5.0), phosphate buffer (pH 6.0 to 7.0), Tris·HCl buffer (pH 7.5 to 8.5), and borate buffer (pH 9 to 10), each at 0.05 *M*.

Peptidase activity was assayed at 30 to 80°C in 0.05 *M* Tris·HCl buffer (pH 7.5). To estimate heat stability, the enzyme was preincubated at 60 or 70°C for 15 and 30 min. Solutions were then cooled in an ice bath, and the remaining activity was determined at 50°C.

Effect of metal ions and chemical reagents. A mixture (80 μl) containing 10 μl of purified enzyme solution and the indicated concentration of each reagent or divalent cation (Tables 2 and 3) (Sigma Chemical Co.) in 0.05 *M* Tris·HCl buffer (pH 7.5) was preincubated for 15 min at 25°C. Residual enzyme activity was assayed by the addition of the substrate, and hydrolysis was estimated by the cadmium ninhydrin method. The enzyme reactivation ability of phenanthroline-inhibited dipeptidase was determined by the addition of different cations (final concentration 1 m*M*) to the enzyme solution that had previously been incubated with phenanthroline (1 m*M*) without dialysis.

Substrate specificity studies. The activity of the purified enzyme was assayed with several dipeptides and tripeptides by the cadmium ninhydrin method. Activity toward *p*-nitroanilide derivatives was measured by the method of Wohlrab and Bockelmann (27). An appropriate amount of enzyme was incubated for 15 min with 2 m*M* substrate solution. Release of nitroaniline (pH 7.5 at 50°C) was measured continuously at 410 nm.

Kinetics studies. The kinetics of the purified dipeptidase toward Leu-Leu and Phe-Ala was determined by the coupled enzyme reaction, using a range of substrate concentration from 0.05 to 4 m*M*. After 8 min of preincubation, substrate was added at different concentrations and hydrolysis was monitored continuously. The data on kinetics were presented in terms of the Hill plot.

RESULTS

Enzyme Purification

Specific activity, degree of purification, and yield at each purification step is summarized in Table 1. The initial heat treatment offered an efficient means of partial purification; thus, 66% of total protein was removed without any loss of the initial activity. In the first chromatographic step, TSK butyl hydrophobic

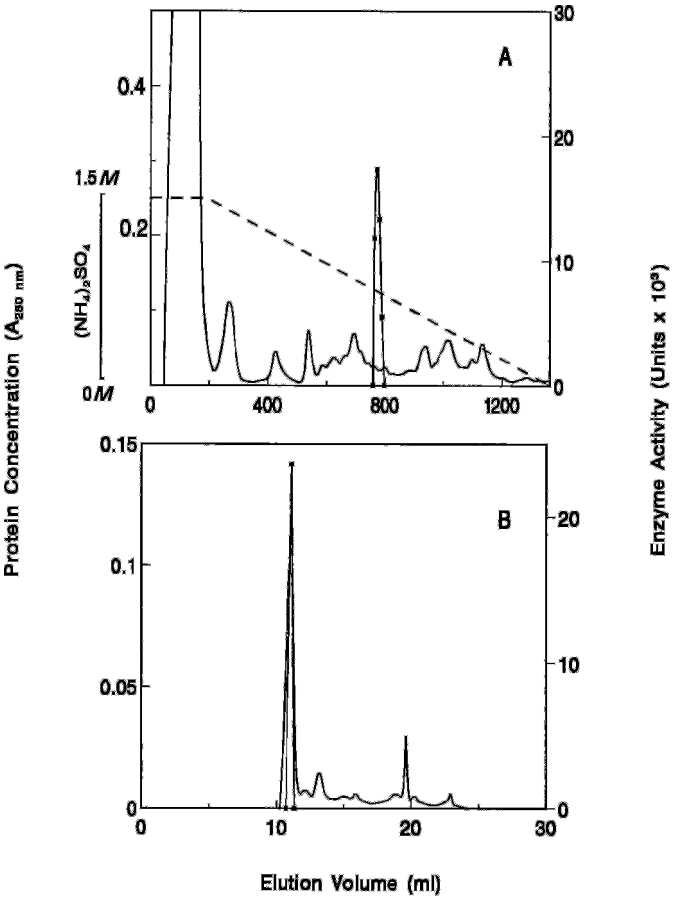

Figure 1. Chromatographic elution profiles of dipeptidase of *Lactobacillus casei* ssp. *casei* IFPL 731 using A) hydrophobic interaction chromatography or B) gel filtration chromatography. Protein at A_{280} (—); gradient used (– –); expressed as nanomoles of Leu-Leu hydrolyzed per minute (■).

interaction chromatography, dipeptidase eluted at 0.75 *M* $(NH_4)_2SO_4$ (Figure 1a). The enzyme was further purified by chromatography on a Mono Q ion-exchange column. Although the enzyme did not bind to the column under our chromatographic conditions, this step resulted in an almost purified enzyme preparation. Some contaminating proteins with low molecular mass in the fraction from Mono Q were removed by gel filtration chromatography on the Superose 12 column (Figure 1b). The active fraction from gel filtration gave a single protein band in SDS-PAGE. By this procedure, the enzyme was enriched about 400-fold; recovery from the crude extract was 20% (Table 1).

Molecular Mass and pI

Molecular mass was estimated to be 46 kDa by SDS-PAGE (Figure 2) and by gel filtration on a Superose column, which suggested that the enzyme exists as a monomer. The pI of the enzyme was estimated to be 4.7 by isoelectric focusing on the Phast system (results not shown).

Effect of Temperature and pH

The enzyme showed a broad temperature optimum in the range 60 to 75°C; at >75°C, enzyme activity decreased sharply (Figure 3). Temperature stability studies confirmed that the enzyme was quite stable at high temperature; 100% of activity remained after preincubation for 30 min at 60°C, but preincubation at 70°C during 15 min decreased the activity by 90%.

The optimum pH for dipeptidase activity was pH 7.5 (Figure 3). At pH values <6, no activity was detected.

Effect of Different Chemical Reagents on Enzyme Activity

The effect of various chemical reagents and metal ions on dipeptidase activity is shown in Table 2. Enzyme activity was strongly activated by Co^{2+} (1 m*M*) and Mn^{2+} (1 m*M*), but Cu^{2+} (1 m*M*) and Fe^{3+}

TABLE 2. Effect of various chemical reagents and metal ions on dipeptidase activity.

Chemical reagent or metal ion	Concentration	Relative activity[1]
	(m*M*)	(%)
β-Mercaptoethanol	1	50
Iodoacetic acid	1	98
	5	74
DTT[2]	1	19
	5	8
$[Fe(CN_6)]^{3-}$	1	63
PMSF[3]	1	108
p-Hydroxymercuri-benzoic acid	1	111
	5	9
Aprotinin	1	97
E64	0.01	127
EDTA	1	51
	5	31
Phenanthroline	1	6
Co^{2+}	1	220
Mn^{2+}	1	235
Zn^{2+}	0.01	131
	0.1	100
	1	16
Cu^{2+}	1	0
K^+	1	111
Fe^{3+}	1	1
Na^+	1	181
	125	47

[1]Activity is expressed as percentage relative to the control.

[2]Dithiothreitol.

[3]Phenylmethylsulfonylfluoride.

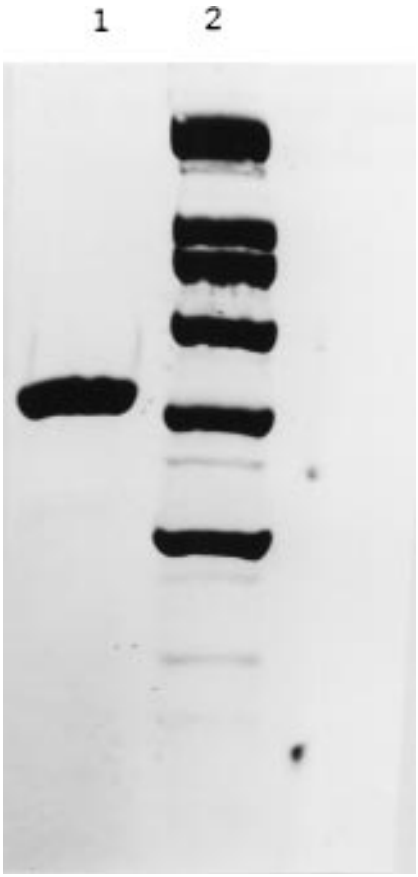

Figure 2. The SDS-PAGE of purified dipeptidase. Lane 1, gel filtration fraction (5.1 μg); lane 2, molecular mass markers (top to bottom): myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa).

(1 m*M*) had an inhibitory effect. Both Zn^{2+} and Na^{+} had a stimulatory effect at concentrations of 0.01 and 1 m*M*, respectively. However, higher concentrations of Zn^{2+} (1 m*M*) and Na^{+} (125 m*M*) inhibited enzyme activity. Metal-chelating agents, such as phenanthroline, markedly inhibited enzyme activity at 1 m*M*; the enzyme was found to be less sensitive to EDTA. After treatment with 1 m*M* phenanthroline, dipeptidase activity was partially restored by addition of 1 m*M* Co^{2+} or Mn^{2+} (Table 3). All metals were added as chlorides to prevent any influence from anions. The results indicate that a metal ion is essential for catalytic activity.

Enzyme activity was not affected by serine protease inhibitors (phenylmethylsulfonylfluoride or aprotinin) or by cysteine protease inhibitors (E-64). Reducing agents such as dithiothreitol and β-mercaptoethanol had an inhibiting effect on enzyme activity at concentrations of 1 and 5 m*M*, and attempts to restore the initial activity inhibited by dithiothreitol with the oxidizing agent $[Fe(CN_6)]^{3-}$ were unsuccessful. The sulfhydryl group reagent, *p*-hydroxymercuribenzoic acid (5 m*M*), also had an inhibitory effect, but iodoacetic acid (5 m*M*) had only a slight inhibitory effect. These results indicate either the specific involvement of sulfhydryl groups in catalysis or the creation of steric hindrance near the active site.

Substrate Specificity

Table 4 shows the relative rates of hydrolysis of various peptides by the purified enzyme. The enzyme was unable to cleave amino acid nitroanilides or tripeptides. Among dipeptides, Leu-Leu and His-Ala were hydrolyzed at the highest rate. The enzyme was active on various dipeptides with hydrophobic amino acid residues (Ala-Val, Ala-Phe, Phe-Leu, Ala-Ala, Phe-Ala), but was less active or not active on dipeptides containing a proline residue. Peptides with a positive charge on the N-terminal amino acid (His-Ala) and peptides containing sulfur were also hydro-

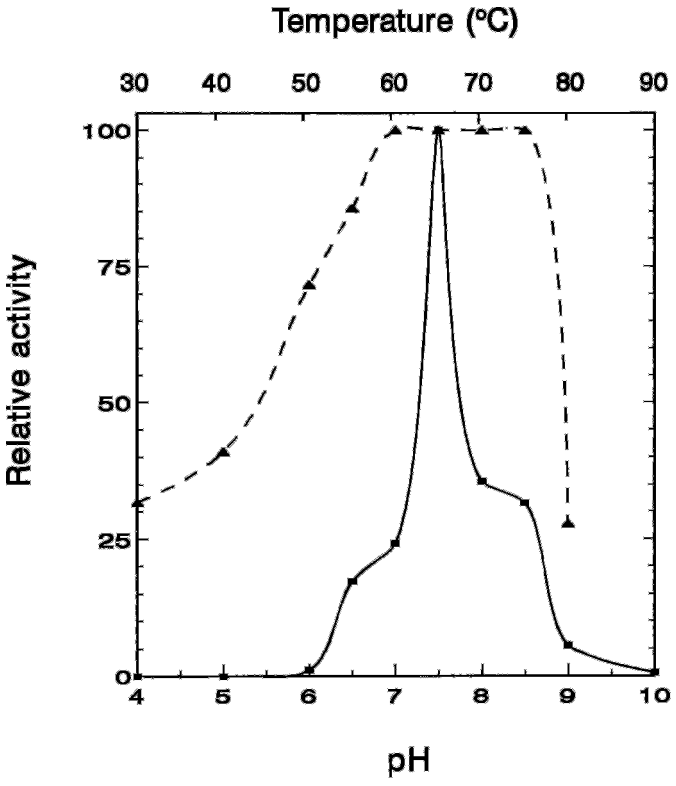

Figure 3. Effect of temperature (▲) and pH (■) on the activity of the purified dipeptidase toward Leu-Leu. Activities are expressed relative to the optimal activity.

TABLE 3. Reactivation of the phenanthroline treated dipeptidase by divalent cation.

Metal ion (1 m*M*)	Relative activity of phenantroline (1 m*M*) (%)
None[1]	9
Co^{2+}	55
Mg^{2+}	0
Mn^{2+}	43
Zn^{2+}	6
Ca^{2+}	7

[1]Residual dipeptidase activity after 15 min of incubation with 1 m*M* phenanthroline. Samples that were not treated by phenanthroline are taken as 100%.

lyzed (Ala-Met, Phe-Met). The N-terminal and second amino acid residues of the dipeptide appeared to be important for the dipeptidase activity.

Kinetics Studies

Studies of the kinetics of Phe-Ala and Leu-Leu revealed an anomalous enzyme behavior. Figure 4 shows the Hill plot for Phe-Ala and Leu-Leu. The cooperative index for Phe-Ala was calculated to be 20 compared with the theoretical value for Michaelis-Menten kinetics of 81. The Hill constant, calculated from the slope of the regression line, was 1.3, which indicates a positive cooperativity. However, at concentrations of Phe-Ala >1 m*M*, enzyme inhibition was strong. The Hill plot for Leu-Leu reflected an intermediate negative cooperative step that corresponds to an intermediary plateau region that was observed between 0.1 to 0.4 m*M* in the saturation curve of Leu-Leu.

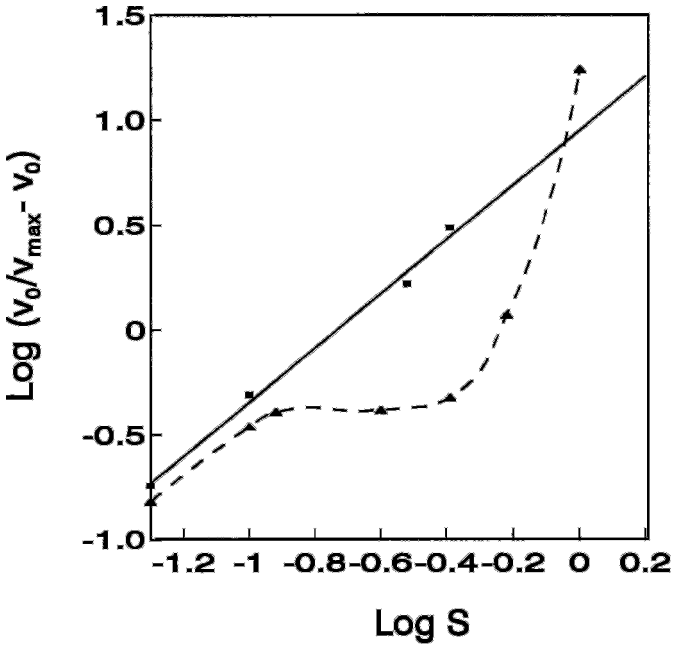

Figure 4. Hill plots of dipeptidase activity toward Phe-Ala (■) and Leu-Leu (▲). Substrate concentration (S) and enzyme activity (V) were expressed in millimolar concentrations and micromoles per milligram per minute, respectively.

The apparent Michaelis constants, calculated from direct plot, for Phe-Ala and Leu-Leu were 0.2 and 0.6 m*M*, respectively, which suggests higher affinity for Phe-Ala than for Leu-Leu.

TABLE 4. Relative activities of the dipeptidase of *Lactobacillus casei* ssp. *casei* IFPL 731 toward various peptides.

Substrate	Relative activity[1] (%)	Substrate	Relative activity (%)
Leu-Leu	100	Phe-Leu	2
His-Ala	88	Leu-Pro	0
Ala-Val	37	Ala-Pro	0
Ala-Met	33	Pro-Phe	0
Phe-Ala	33	Val-Asp	0
Ala-Ala	26		
Lys-Ala	25	Arg-Pro-*p*-NA[2]	0
Ala-Phe	23	Lys-*p*-NA	0
Leu-Gly	21	Pro-*p*-NA	0
Phe-Met	21	Leu-*p*-NA	0
Phe-Leu	19		
Val-Ala	10	Pro-Gly-Gly	0
Phe-Pro	8	Ala-Leu-Gly	0
Leu-Tyr	7	Leu-Gly-Phe	0

[1]Activity against Leu-Leu was taken as 100%.

[2]*p*-NA = *p*-Nitroanilide.

DISCUSSION

This paper describes the purification and characterization of a dipeptidase from *Lb. casei* ssp. *casei* IFPL 731. Although a number of metal-dependent dipeptidases have been isolated and characterized from various lactic acid bacteria [*Lactococcus lactis* ssp. *cremoris* strain H61 (12) and strain Wg2 (25), citrate-utilizing *Lactococcus lactis* ssp. *lactis* (5), *Streptococcus thermophilus* (20), *Lactobacillus delbrueckii* ssp. *bulgaricus* (27), *Lactobacillus casei* NCDO 151 (8), and *Lactobacillus helveticus* SBT2171 (23)], various features of the dipeptidase of *Lb. casei* IFPL 731 (e.g., thermal stability, kinetic behavior, and temperature optimum) differ from the dipeptidases purified and characterized so far.

The purified enzyme had a molecular mass of 46 kDa. Similar values have been reported for dipeptidase from *Lc. lactis* ssp. *cremoris* Wg2, citrate-utilizing *Lc. lactis* ssp. *lactis*, *S. thermophilus*, *Lb. delbrueckii* ssp. *bulgaricus*, and *Lb. helveticus* (49 to

51 kDa), which are monomeric. However, the dipeptidase of *Lc. lactis* ssp. *cremoris* H61 had a molecular mass of 100 kDa by conventional gel filtration. No data were given for *Lb. casei* NCDO 151 dipeptidase.

The temperature optimum for the dipeptidase from *Lb. paracasei* IFPL 731 was higher than those of other dipeptidases studied. Although previously reported dipeptidases were more active at 50°C—with the exception of *Lb. casei* NCDO 151, which was most active at 30°C—the enzyme reported here had an optimum temperature between 60 and 75°C. This enzyme also appeared to be quite resistant to heat, and therefore the thermal stability of the enzyme (30 min at 60°C) was used as part of the purification procedure. Only the dipeptidases of *S. thermophilus* were more thermostable, retaining 75% of their residual activity at 70°C. Our enzyme rapidly lost activity on preincubation at 70°C, but maximum enzyme activity was still found at 75°C, which suggests that the presence of the substrate in the enzyme solution had a stabilizing effect. The optimal pH of the dipeptidase was 7.5, which compares well with other dipeptidases, which had optima ranging from pH 7.0 to 8.0.

The effect of inhibitors showed that the dipeptidase in this study is a metalloenzyme, as are all previously reported dipeptidases. Unlike other dipeptidases, the enzyme was more sensitive to phenanthroline (specific inhibitor for Zn^{2+} metalloproteases) than to EDTA. However, the activity of phenanthroline-inhibited dipeptidase was restored by addition of Co^{2+} or Mn^{2+}; results were similar for the EDTA-treated dipeptidase of *Lactococcus*, *Lactobacillus*, and *Streptococcus* spp. The dipeptidase from *L. helveticus* was highly stimulated by Zn^{2+}, and the activity lost by treatment with phenanthroline was partially restored with Zn^{2+}. In contrast to *Lb. delbrueckii* ssp. *bulgaricus* dipeptidase, sulfhydryl and reducing agents inhibited the dipeptidase activity of *Lb. paracasei* IFPL 731. Similar effects have been reported for enzymes from *Lc. lactis* ssp. *cremoris* strains H61 and Wg2 and *Lb. helveticus*. This finding suggests that the hydrolyzing activity of the dipeptidase from *L. casei* IFPL 731 may depend on the enzyme being in an oxidized or a reduced state. However, enzyme activity inhibited by dithiothreitol could not be restored by $[Fe(CN_6)]^{3-}$.

Studies of substrate specificity showed that the dipeptidase was active toward a wide range of dipeptides but could not hydrolyze tripeptides or amino acid nitroanilides. Like other dipeptidases from *S. thermophilus, Lc. lactis* ssp. *cremoris* strains H61 and Wg2, *Lb. delbrueckii* ssp. *bulgaricus*, and *L. helveticus*, the enzyme was quite active toward dipeptides with a hydrophobic N-terminal amino acid.

The most striking feature of the enzyme was its kinetic behavior. This behavior contrasted with other dipeptidases, which followed Michaelis-Menten saturation kinetics, exhibiting a hyperbolic curve with no inflection points. The purified enzyme in the present study seemed to be the allosteric type, because it showed a mixture of positive and negative cooperativity for the successive binding of molecules of the same substrate. Frieden (10) showed that bursts or lags of enzyme activity can occur when a single-substrate enzyme exits two conformational states that have different substrates affinities. In addition, Rübsamen et al. (22) postulated a molecular model that was based on conformational changes induced by the ligand for the interpretation of nonhyperbolic kinetics.

CONCLUSIONS

Peptidases are necessary for optimal bacterial growth because they provide the cells with essential amino acids. Peptidases are also responsible for the degradation of bitter peptides in cheese and can produce the free amino acids that are precursors of aromatic compounds. Because the dipeptidase from *Lb. casei* IFPL 731 is sensitive to low pH and NaCl, this dipeptidase is unlikely to remain active in the cheese matrix after lysis. However, unlike lactococci, mesophilic lactobacilli lyse less readily and may be able to maintain an intracellular pH ~6.0 even when the pH outside was <4.5 (20), which implies a possible role for the dipeptidase in the course of cheese ripening.

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REFERENCES

1 Arora, G., and B. H. Lee. 1992. Purification and characterization of aminopeptidase from *Lactobacillus casei* ssp. *casei* LLG. J. Dairy Sci. 75:700.

2 Arora, G., and B. H. Lee. 1994. Purification and characterization of an aminopeptidase from *Lactobacillus casei* subsp. *rhamnosus*. Biotechnol. Appl. Biochem. 19:179.

3 Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248.

4 Broome, M. C., and M. W. Hickey. 1991. Peptidase activity of non-starter lactobacilli. Aust. J. Dairy Technol. 46:19.

5 Desmazeaud, M. J., and J. Zevaco. 1977. General properties and substrate specificity of an intracellular soluble dipeptidase from *Streptococcus diacetylactis*. Ann. Biol. Anim. Biochim. Biophys. 17:723.
6 Doi, E., D. Shibata, and T. Matoba. 1981. Modified colorimetric ninhydrin methods for peptidase assay. Anal. Biochem. 118: 173.
7 El-Abboudi, M., M. El-Soda, S. Pandian, R. E. Simard, and N. F. Olson. 1992. Purification of X-prolyl dipeptidyl aminopeptidase from *Lactobacillus casei* subspecies. Int. J. Food Microbiol. 15: 87.
8 El-Soda, M., M. J. Desmazeaud, and J.-L. Bergère. 1978. Peptide hydrolases of *Lactobacillus casei*: isolation and general properties of various peptidase activities. J. Dairy Res. 45:445.
9 Fontecha, J., C. Peláez, M. Juárez, T. Requena, R. Gómez, and M. Ramos. 1990. Biochemical and microbiological characteristics of artisanal hard goat's cheese. J. Dairy Sci. 73:1150.
10 Frieden, C. 1964. Kinetic aspect of regulation of metabolic process. J. Biol. Chem. 245:5788.
11 Habibi-Najafi, M. B., and B. H. Lee. 1995. Purification and characterization of proline iminopeptidase from *Lactobacillus casei* ssp. *casei* LLG. J. Dairy Sci. 78:251.
12 Hwang, I-K., S. Kaminogawa, and K. Yamauchi. 1981. Purification and properties of a dipeptidase from *Streptococcus cremoris*. Agric. Biol. Chem. 45:159.
13 Khalid, N. M., and E. H. Marth. 1990. Lactobacilli—their enzymes and role in ripening and spoilage of cheese: a review. J. Dairy Sci. 73:2669.
14 Kunji, E.R.S., I. Mierau, A. Hagting, B. Poolman, and W. N. Konings. 1996. The proteolytic system of lactic acid bacteria. Antonie Leeuwenhoek 70:187.
15 Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) 227: 680.
16 Law, B. A., and J. Kolstad. 1983. Proteolytic system in lactic acid bacteria. Antonie Leeuwenhoek 49:225.
17 Lee, B. H., S. Haché, and R. E. Simard. 1986. A rapid method for differentiation of dairy lactic acid bacteria by enzyme systems. J. Ind. Microbiol. 1:209.
18 Nannen, N. L., and R. W. Hutkins. 1991. Intracellular pH effects in lactic acid bacteria. J. Dairy Sci. 74:741.
19 Peterson, S. D., and R. T. Marshall. 1990. Nonstarter lactobacilli in Cheddar cheese: a review. J. Dairy Sci. 73:1395.
20 Rabier D., and M. J. Desmazeaud. 1973. Inventaire des differentes activités peptidasiques intracellulaires de *Streptococcus thermophilus*. Purification et propriétés d'une dipeptide-hydrolase et d'une aminopeptidase. Biochimie (Paris) 55:389.
21 Requena, T., M. A. De la Fuente, P. Fernández de Palencia, M. Juárez, and C. Peláez. 1992. Evaluation of a specific starter for the production of semi-hard goat's milk cheese. Lait 72:437.
22 Rübsamen, H., R. Klhandker, and H. Witzel. 1974. Sigmoid kinetics of the monomeric ribonuclease I due to ligand-induced shifts of conformation equilibria. Hoppe-Seyler's Z. Physiol. Chem. 355:687.
23 Tan, P.S.T., M. Sasaki, B. W. Bosman, and T. Iwasaki. 1995. Purification and characterization of a dipeptidase from *Lactobacillus helveticus* SBT 2171. Appl. Environ. Microbiol. 61: 3420.
24 Trépanier, G., R. E. Simard, and B. H. Lee. 1991. Effect of added lactobacilli on composition and texture of Cheddar cheese during accelerated maturation. J. Food Sci. 56:696.
25 van Boven, A., P.S.T. Tan, and W. N. Konings. 1988. Purification and characterization of a dipeptidase from *Streptococcus cremoris* Wg2. Appl. Environ. Microbiol. 54:43.
26 Vescovo, M., S. Torriani, G. Scolari, and L.M.T. Dicks. 1995. *Lactobacillus casei* and related species: a review. Ann. Microbiol. Enzymol. 45:51.
27 Wohlrab, Y., and W. Bockelmann. 1992. Purification and characterization of a dipeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus*. Int. Dairy J. 2:345.