Characterization of the Aminopeptidase System from *Lactobacillus casei* Subsp. *casei* IFPL 731

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The cell-free extract of *Lactobacillus casei* subsp. *casei* IFPL 731 was subjected to ammonium sulfate precipitation, hydrophobic interaction chromatography, two steps of anion-exchange chromatography, and gel filtration. Six peaks with aminopeptidase (Ap) activity toward *p*-nitroanilide (*p*NA) derivatives were found (Ap I to Ap VI). Ap I, Ap III, Ap IV, and Ap V hydrolyzed preferentially Lys-*p*NA and were identified as metalloenzymes; only Ap IV was strongly activated by the sulfydryl group reagent *p*-hydroxymercuribenzoic acid. Peak Ap II cleaved Leu-*p*NA, but not Lys-*p*NA or Arg-*p*NA, and it was not affected by inhibitors of metallopeptidases but by *p*-hydroxymercuribenzoic acid. Ap VI preferentially hydrolyzed Leu-*p*NA, and it was a metalloenzyme. Ap III was purified to homogeneity and identified as an aminopeptidase N with a molecular mass of 95 kDa by SDS−PAGE as well by gel filtration. Ap IV was also purified and seems to be tetrameric with a molecular mass of 30 kDa.

Keywords: *Aminopeptidase system; Lactobacillus casei; characterization*

INTRODUCTION

Among lactic acid bacteria, lactobacilli are the most fastidious microorganisms, showing high extensive amino acid requirements for growth. This implies the development of a proteolytic system that hydrolyzes the milk casein and provides the cell with these essential substrates. In fact, it has been demonstrated that lactobacilli exhibit greater peptidase activities than lactococci (Hickey *et al*., 1983). Besides, lactobacilli are often the dominant flora during the late stages of cheese ripening, and therefore they may play a crucial role in the secondary proteolysis of cheese and in the development of its characteristic flavor (Hickey *et al*., 1983; Ezzat *et al*., 1986; Lee *et al*., 1986).

Analysis of heat inactivation kinetics and effect of inhibitors on the enzymatic activity pointed out the existence of four aminopeptidases in *Lactobacillus bulgaricus* (Atlan *et al*., 1989). In addition, electrophoretic analysis has detected three aminopeptidases in different strains of *L. casei* (El Soda and Ezzat, 1993). Different aminopeptidases of *L. delbrückii* subsp. *lactis* (PepN, PepL, PepC, PepI, PepV and PepX) and *L. helveticus* (PepC, PepN, PepXP, PepPN, and PepDI) have been identified by cloning and sequencing their corresponding genes (Kok and Venema, 1995).

The authors of the first systematic study of peptidases from *Lactobacillus casei*, El Soda *et al.* (1978a,b), detected an endopeptidase and three exopeptidases in the intracellular fraction of *L. casei* NCDP 151. In subsequent studies, others found not only those but also tripeptidase (Abo-Elnaga and Plapp, 1987; Arora and Lee, 1990; El Abboudi *et al*., 1991, 1992a) and arylpeptidylamidase activity in cell-free extracts of *L. casei* (El Soda and Ezzat, 1993). Aminopeptidase N of *L. casei* subsp. *casei* LLG and *L. casei* subsp. *rhamnosus* (Arora and Lee, 1992, 1994) have been purified, as well as PepX activity of *L. casei* subsp. *casei* UL21 and LLG (El-Abboudi *et al*., 1992b; Habibi-Najafi and Lee, 1994). However, there have been very few studies on purification of component enzymes of the peptidase system of *L. casei*.

In previous studies at our laboratory, a high level of aminopeptidase, PepX, and dipeptidase activity was found in the cell-free extract of *L. casei* IFPL 731 isolated from Spanish goat's milk cheese (Requena *et al*., 1993). A prolidase and a dipeptidase were recently purified from this strain (Fernández-Esplá *et al*., 1997; Fernández-Esplá and Martín-Hernández, 1997). This paper describes the partial characterization of the aminopeptidase system of *L. casei* IFPL 731.

MATERIALS AND METHODS

Microorganism, Culture Conditions, and Harvesting. *L. casei* subsp. *casei* IFPL 731 was subcultured twice overnight at 30 °C in MRS broth (Oxoid, Basingstoke, England) before being used to inoculate fresh medium (2% inoculum). Cells from 5 L of the culture incubated at 30 °C were harvested by centrifugation (7500*g*, 20 min, 4 °C) at late exponential growth phase [approximately 10^9 colony forming units (cfu) mL^{-1}]. The pellet was washed with 50 mM sodium phosphate buffer, pH 7.0, and stored at −80 °C until preparation of the cell-free extract.

Preparation of the Cell-Free Extract. For disruption, the cells were resuspended in 70 mL of 20 mM Tris-HCl, pH 7.5, mixed with glass beads (diameter 0.15−0.25 mm; Sigma Chemical Co., St. Louis, Mo) (1:1 w v^{-1}) and shaken (four times for 4 min each at 4 °C) in a Mini Blend (Sunbeam-Oster, Miami, FL). After centrifugation (17000*g*, 20 min, 4 °C), the clear supernatant was incubated with RNase (2.5 μg mL^{-1}) and DNase (5 μg mL^{-1}) (Boehringer Manheim Biochemica, Manheim, Germany) for 30 min at room temperature. This fraction was designated cell-free extract (CFE).

Fractionation of the Aminopeptidase (AP) Activities. *Ammonium Sulfate Precipitation.* CFE was fractionated with ammonium sulfate (AS) in two steps (31 and 44% w v^{-1}). The precipitated protein at 31% w v^{-1} AS was discarded, and the aminopeptidase activity was collected by centrifugation (20000*g*, 20 min, 4 °C) at 44% w v^{-1} of this salt. The pellet was redissolved in 12.5 mL of 20 mM Tris-HCl, pH 7.5. This fraction was designated AS fraction.

Hydrophobic Interaction Chromatography (HIC). AS was added to the AS fraction to a final concentration of 1.5 M. The

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sample was applied to a Fractogel TSK butyl-650 S (Merck, Darmstadt, Germany) column (180 mm × 26 mm) equilibrated with 1.5 M AS in 20 mM Tris-HCl, pH 7.5. Bound proteins were eluted at a flow rate of 2.5 mL min^{-1} in a decreasing AS gradient (1.5 to 0 M) in the same buffer. Four AP active fractions, designated HIC I, HIC II, and HIC IV (each one, 20 mL) and HIC III (30 mL), were eluted and desalted by passing them through Sephadex G-25 PD-10 columns (Pharmacia Biotech, Uppsala, Sweden) equilibrated with the same buffer.

Anion-Exchange Chromatography. The HIC desalted fractions were applied separately to a Mono Q HR 5/5 (Pharmacia) column, equilibrated with 20 mM Tris-HCl, pH 7.5. Bound proteins eluted at a flow rate of 1 mL min^{-1} in a linear NaCl gradient (0−1 M) in the same buffer. Since from both HIC II and HIC IV two peaks with AP activity were eluted, a total of six active Ap fractions were obtained (Ap I to Ap VI). After desalting by Sephadex G-25 (Pharmacia) columns, all fractions were subjected to a second anion-exchange chromatography and later desalted in the same conditions as described above. The Ap fractions were concentrated in an ultrafiltration cell (10 mL) (Amicon Danvers, Beverly, MA) using a 10 kDa membrane type Omega (Filtron Technology, Northborough, MA).

Gel Filtration. Portions (200 μL) of concentrated fractions were applied on a Superose 12 HR 10/30 (Pharmacia) column and eluted with 0.1 M Tris-HCl, pH 7.5, at a flow rate of 0.3 mL min^{-1}. The column was calibrated using gel filtration molecular weight standards (MW-GF-200 kit; Sigma).

All steps of purification were carried out at 4 °C during the separation procedure, the enzymatic fractions were stored at −80 °C in the presence of stabilizers [(10% glycerol and 1 mM dithiothreitol (DTT)].

AP Activity. AP activity at each separation step was routinely tested with the substrates Lys-*p*-nitroanilide (Lys-*p*NA) and Leu-*p*NA according to the method described by Exterkate (1984). To study the specificity of the AP fractions, the substrates Arg-*p*NA and Pro-*p*NA were also used (Sigma). The reaction mixture contained 400 μL of sodium phosphate buffer (50 mM, pH 7.0), 50 μL of substrate (1 mM final concentration), and 50 μL of appropriate enzyme solution. Enzyme activity was measured continuously for 10 min at 410 nm and 37 °C by assaying the quantity of *p*-nitroaniline released. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 nmol of *p*-nitroaniline per minute and per milliliter under assay conditions (E_{410} = 8800 M^{-1} cm^{-1}). Specific activity was expressed as units per milligram of protein.

Determination of Protein Concentration. Protein concentration was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin (Sigma) as standard.

Characterization of the AP Activity. The characterization study of the AP activity of *L. casei* IFPL 731 was carried out with the Ap fractions eluted from the second anion-exchange chromatography. Activity was measured using Lys-*p*NA as substrate, except for the Ap II fraction for which Leu-*p*NA was used.

Effect of pH and Temperature. The effect of pH on the activity of Ap fractions was measured in 0.1 M acetate (pH 4.0 and 5.0), 0.1 M sodium phosphate (pH 6.0 and 7.0), 0.1 M Tris-HCl (pH 7.5 and 8.0), and glycine−NaOH (pH 9.0 and 10.0) buffers at 37 °C.

The influence of temperature (at pH 7.0) on the AP activity was determined by measuring the hydrolysis of the substrate in the range of temperature of 25−60 °C.

Effect of Chemical Reagents. A mixture containing 50 μL of the appropriate dilution of Ap fraction and 25 μL of chemical reagent (final concentration = 1 mM) in 375 μL of 50 mM sodium phosphate buffer, pH 7.0, was incubated for 30 min at room temperature. Reaction was initiated by the addition of 50 μL of substrate (final concentration = 1 mM).

SDS−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE under reducing conditions was performed by using the Phast System (Pharmacia) according to the instruction manual. Electrophoresis was carried out with 12.5% polyacrylamide gels and SDS buffer strips (Pharmacia). For staining, Coomassie Brilliant Blue R-250 (Merck) was used. SDS molecular weight markers (molecular mass 14−94 kDa; LMW calibration kit, Pharmacia) were used as reference proteins.

Table 1. Fractionation Procedure Scheme of AP Activity from *L. casei* Subsp. *casei* IFPL 731

separation step	substrate	total act.[a] (units)
CFE	Lys-*p*NA	30860
	Leu-*p*NA	10464
AS precipitation	Lys-*p*NA	9860
	Leu-*p*NA	3248

separation step	substrate	HICI	HIC II	HIC III	HIC IV
TSK-butyl	Lys-*p*NA	<1	1270	4510	984
	Leu-*p*NA	748	1003	500	173

separation step	substrate	Ap II	Ap III	Ap I	Ap IV	Ap V	Ap VI
Mono Q-I	Lys-*p*NA	<1	53	46	3940	90	570
	Leu-*p*NA	552	46	36	485	72.5	1295
Mono Q-II	Lys-*p*NA	<1	14	9	1300	22	25
	Leu-*p*NA	553	12	7	185	18	58

[a] Total activity is expressed as nmol of *p*-nitroaniline released from Lys and Leu-*p*NA substrates per min. [b] TSK-butyl, elution from hydrophobic interaction chromatography (HIC). [c] Mono Q-I and -II, elution from anion-exchange chromatography.

RESULTS AND DISCUSSION

Fractionation of AP Activity. Table 1 shows the separation of AP activity from *L. casei* IFPL 731 measured versus the Lys-*p*NA and Leu-*p*NA substrates. After AS precipitation (31−44% w v^{-1}), >30% of the AP activity was recovered. With hydrophobic interaction chromatography (HIC), four active fractions (HIC I−IV) were eluted at 0.80, 0.60, 0.15, and 0 M AS, respectively. After each HIC fraction was twice loaded onto the anion-exchange chromatography column, a total of six Ap fractions eluted at 0.15 M (Ap III−V), 0.2 M (Ap I and VI), and 0.25 M (Ap II) of the NaCl gradient. SDS−PAGE analysis showed that only the Ap IV fraction resulted in a single band, which suggests that this preparation was homogeneous and had an apparent molecular mass of 30 kDa (Figure 1b). The purification process for this fraction is summarized in Table 2. Separation was continued by applying the six active fractions in a gel filtration column. This step did not improve separation of fractions Ap I, II, V, and VI, and the peaks were not clearly defined. Fraction IV eluted as a well-defined peak at an estimated molecular mass of 120 kDa, which suggested that this enzyme was in its native state in tetramer form. Fraction Ap III was also purified following gel filtration with a molecular mass of 90 kDa. This molecular mass was the same as estimated by SDS−PAGE, indicating that the enzyme was in monomer form (Figure 1a). Purification of fraction Ap III is detailed in Table 2, emphasizing a low purification-fold due to the high instability of this enzyme.

The molecular mass of the Ap IV enzyme (30 kDa) was the same as found for purified aminopeptidases in lactococci (Geis *et al.*, 1985) and lactobacilli (Machuga and Ives, 1984; Wohlrab and Bockelmann, 1994), which were again in multimer form. The molecular mass of Ap III was similar to that observed for AP N (87−97 kDa) of lactic acid bacteria (Kunji *et al.*, 1996).

Characterization of AP Activity. *Effect of pH and Temperature.* As described for most of the general aminopeptidases of lactic bacteria, maximum activity of the enzyme fractions occurred within a pH range of

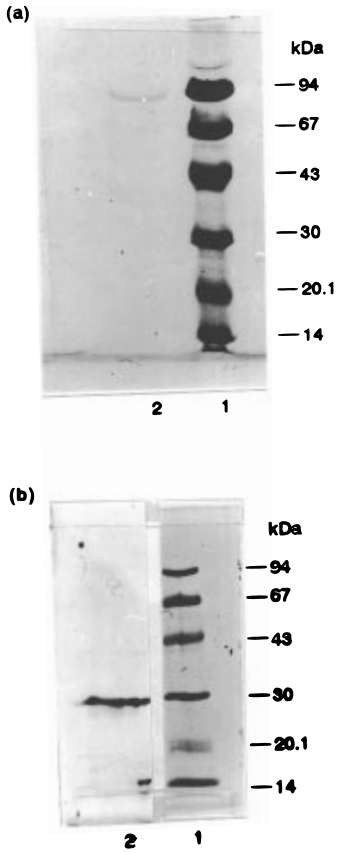

Figure 1. SDS−PAGE of the purified aminopeptidases Ap III and Ap IV. Electrophoresis was performed on an SDS−polyacrylamide gel (12.5% polyacrylamide). (Lane 1, a and b) several reference proteins in kDa; (lane 2) purified Ap III (a) and Ap IV (b).

Table 2. Purification of Ap III and Ap IV Fractions from *L. casei* Subsp. *casei* IFPL 731

purifn step	total protein (mg)	total act.[a] (units)	specific act.[b] (units/mg of protein)	purifn (-fold)	yield (%)
CFE	436	30860	71	1	100
AS precipitation	116	9860	85	1.2	32
Ap III					
TSK-butyl	22.8	1270	56	0.8	4.1
Mono Q-I	0.4	53	132.5	1.8	0.2
Mono Q-II	0.24	14	58.3	0.8	0.04
Superose 12	0.048	13	270	4	0.04
Ap IV					
TSK-butyl	8.48	4510	532	7.5	14.6
Mono Q-I	7.98	3940	494	7	12.7
Mono Q-II	0.37	1300	3513	49.4	4.2

[a] Total activity is expressed as nmol of *p*-nitroaniline released from Lys-*p*NA substrate per min. [b] Specific activity is expressed as total activity per mg of protein.

around 6.5−7.5 and a temperature range of 35−37 °C (Kok and de Vos, 1994; Kunji *et al.*, 1996). Temperature optimum of the Ap I fraction was lower (30 °C) compared to that of Ap II (55 °C).

Effect of Chemical Agents. The effect of a number of chemicals on the AP activity of *L. casei* IFPL 731 is shown in Table 3. Fraction Ap IV exhibited approximately 50% inhibition by phenylmethanesulfonyl fluoride (PMSF), which indicates the importance of serine residues in the activity of this enzyme. Inhibition by PMSF is not a normal characteristic of general aminopeptidases of lactic bacteria (Kunji *et al.*, 1996). On the other hand, AP L from *L. delbrückii* subsp. *lactis* was recently classified as a serine peptidase the gene product of which is predicted to be 35 kDa (Klein *et al.*, 1995).

Table 3. Effect of Chemical Reagents on AP Activity from *L. casei* Subsp. *casei* IFPL 731

	AP activity[a] (%)				
fraction	PMSF	DTT	EDTA	1,10-phenanthroline	PHMB
Ap I	73	98	11	11	206
Ap II	71	92	95	89	87
Ap III	86	97	13	13	171
Ap IV	47	142	57	37	526
Ap V	128	76	28	14	100
Ap VI	86	71	6	14	100

[a] Samples not treated by chemical reagents are taken as 100%. The concentration of inhibitors was 1 mM.

The disulfide group reducer DTT caused roughly 30% inhibition in fractions Ap V and Ap VI. At the same time there was a noticeable increase (approximately 140%) in activity of fraction Ap IV, indicating that the reduced disulfide groups stabilized the catalytic action of the enzyme contained in the fraction. DTT has also been found to have an inhibiting effect in AP N of *L. casei* subsp. *casei* LGG (Arora and Lee, 1992) but an activating effect in AP N of *L. lactis* subsp. *cremoris* Wg2 (Tan and Konings, 1990) and *L. delbrückii* subsp. *bulgaricus* B14 (Bockelmann *et al.*, 1992). The findings in the literature suggest that the state of this kind of enzyme (oxidized or reduced) is an important factor in its activity.

Metal chelant agents ethylenediaminetetraacetic acid (EDTA) and 1,10-phenanthroline strongly inhibited the activity of fractions Ap I, III, V, and VI, thus identifying them as metalloenzymes. The activity of Ap IV was reduced by about 40% versus EDTA and by 60% versus 1,10-phenanthroline at the given concentration (1 mM), suggesting that divalent cations may play an important role in the catalytic activity of this enzyme. This behavior is very different from that reported in the PepL mentioned above, which was not affected by the action of chelant agents (Klein *et al.*, 1995). On the other hand, fraction Ap II was not inhibited by the chelant agents, suggesting that the AP activity of this fraction is not metal-dependent.

The fractions were not affected in the presence of the thiol−protease inhibitor *p*-hydroxymercuribenzoic acid (PHMB) at a concentration of 1 mM. However, a higher concentration (5 mM) of this agent inhibited 95% of activity in fraction Ap II, indicating that AP belongs to the thiol group peptidases. Also, PHMB was found to activate fractions Ap I, III, and IV, raising activity by 526% in the last of these. The absence of inhibition by PHMB has also been noted in all of the aminopeptidases recorded in the literature, with the exception of cystein−AP C, which is inhibited by PHMB (Kok and de Vos, 1994; Kunji *et al.*, 1996). These results strongly suggest that the activity of the Ap II fraction could correspond to an AP C.

Specificity of the Substrate. The specificity of the different fractions toward various *p*NA derivative substrates is shown in Table 4. All of the fractions preferentially hydrolyzed the Lys-*p*NA substrate, except for Ap II and VI which exhibited a preference for the Leu-*p*NA substrate. The Arg-*p*NA substrate was hydrolyzed in varying degrees by all of the fractions except Ap II, which showed no apparent specificity versus derivatives of *p*-nitroanilide with positive-charge amino acids, such as Lys and Arg in its N-terminal end.

Table 4. Substrate Specificity of AP Activity from *L. casei* Subsp. *casei* IFPL 731

	relative act.[a] (%)			
fraction	Lys-*p*NA	Leu-*p*NA	Pro-*p*NA	Arg-*p*NA
Ap I	100	78	17	76
Ap II	2	100	12	1
Ap III	100	87	18	58
Ap IV	100	20	39	39
Ap V	100	82	9	36
Ap VI	43	100	17	25

[a] Expressed as percentage of maximal activity measured for each Ap fraction.

Activity toward the Pro-*p*NA substrate was low (<20%) in all cases except for fraction Ap IV, where there was 40% activity. Although the specificity of peptide hydrolysis with proline residues is not a common characteristic of the general type of aminopeptidases, such specificity has also been reported in the AP described by Wohlrab and Bockelmann (1994) in *L. delbrückii* subsp. *bulgaricus* B14. Considering that this enzyme has a molecular weight similar to that of Ap IV, it seems probable that the same type of enzyme is involved.

The present work shows that *L. casei* subsp. *casei* IFPL 731 possesses a very elaborated AP system consisting of six active fractions with high specificity toward Lys- and Leu-*p*NA substrates. This AP system is very important with regard to using this strain as an adjunct in semihard cheeses, since it could both prevent bitterness, a frequently reported defect in different cheese varieties, and help enhance development of desired flavors in cheese.

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