Two cell-wall-associated aminopeptidases from *Lactobacillus helveticus* and the purification and characterization of APII from strain ITGL1

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(Received 25 November 1992; revised 11 February 1993; accepted 26 March 1993)

Lactobacillus helveticus ITGL1 is able to hydrolyse many amino-acyl and dipeptidyl-*p*-nitroanilides. Analysis of heat inactivation kinetics, metal ion and protease inhibitor effects, and the subcellular location of aminopeptidase activities in both the parental strain and mutants deficient in lysyl-*p*-nitroanilide hydrolysis, led to the characterization of two cell-wall-associated aminopeptidases, APII and APIV. APII, which catalysed L-lysine *p*-nitroanilide hydrolysis, was purified about 28-fold to homogeneity from cell-wall extracts of *L. helveticus* ITGL1 and characterized. The purified enzyme appeared to be monomeric, with a molecular mass of 97 kDa. Aminopeptidase activity was greatest at pH 6.5 and 50 °C. APII was completely inhibited by bestatin, chelating agents such as EDTA or 1,10-phenanthroline and the divalent cations Zn²⁺ and Cu²⁺. The activity of the EDTA-treated enzyme was restored by Co²⁺, Ca²⁺ or Mn²⁺. Although APII was able to degrade several dipeptides and tripeptides with hydrophobic N-terminal amino acid (Leu, Ala), it was inactive on peptides containing Pro or Gly, and may thus contribute to the development of cheese flavour by processing bitter peptides.

Introduction

Lactic acid bacteria play important roles in food production and preservation, especially in the dairy industry. These bacteria are unable to synthesize most amino acids and require an exogenous nitrogen source for growth (Thomas & Pritchard, 1987; Law & Koshlsted, 1983). The concentrations of free amino acids in milk are not sufficient to support significant cell growth. Therefore, lactococci depend on proteolytic systems that consist of several proteinases and aminopeptidases to degrade milk caseins into assimilable peptides and free amino acids (Kok, 1990; Laan *et al.*, 1989). The cell-wall-bound proteinases of lactic acid bacteria perform the first steps in the hydrolysis of casein (Kok, 1990; Kok & Venema, 1988). Subsequent steps in casein catabolism are catalysed by peptidases, and make possible the degradation into smaller products of relatively large peptide fragments released by the proteinases (Monnet *et al.*, 1986, 1989). An understanding of the

roles played by peptidases in casein degradation requires the isolation and biochemical characterization of purified enzymes (for review see Kok, 1990).

The use of lactobacilli such as *Lactobacillus helveticus* together with streptococci has been shown to shorten the time of cheese ripening and thus reduce costs (Bartels *et al.*, 1987). This has been associated with an increase in the level of some amino acids and peptides that give the fermentation products their characteristic organoleptic properties. The present investigation was undertaken to analyse the proteolytic system of *L. helveticus*.

L. helveticus is able to hydrolyse α_s and β -caseins; it contains high levels of general aminopeptidase (AP), proline iminopeptidase (Pro-IP) and aminopeptidase-P activities, but no carboxypeptidase activity (Hickey *et al.*, 1983). A prolyl-dipeptidyl aminopeptidase (Khalid & Marth, 1990*a*) and an intracellular aminopeptidase (Khalid & Marth, 1990*b*) have been purified from *L. helveticus* CNRZ 32, and characterized. Another aminopeptidase has been purified from *L. helveticus* LHE-511 (Miyakawa *et al.*, 1992).

In the present study, we have developed a procedure for the release of cell wall proteins from *L. helveticus* ITGL1 (industrial strain). Heat inactivation studies and differential inhibition by EDTA and metal ions indicated

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Abbreviations: pNA, *para*-nitroanilide, AP, aminopeptidase, DPAP, dipeptidyl aminopeptidase.

the existence of two aminopeptidases, which we named APII and APIV. We also report the analysis of mutants deficient in APII and the purification and the properties of cell-wall-associated APII.

Methods

Organism and culture conditions. *Lactobacillus helveticus* ITGL1 was supplied by the Institut Technique du Gruyère, La Roche sur Foron, France. *L. helveticus* was grown at 40 °C without shaking in 125 ml flasks containing 100 ml culture medium. Rich peptide medium was MRS broth (Difco; De Man *et al.*, 1960). Reconstituted 10% (w/v) skim milk solutions from skim milk powder (Elle et Vire, Union Laitière Normande, Condé-sur-Vire, France) were centrifuged (15000 g, 15 min, 6 °C) and then autoclaved at 110 °C for 15 min. When MRS medium was used, β -galactosidase synthesis was induced by adding 1 mM-IPTG.

Subcellular fractionation. (i) *Total cellular extracts.* Total cellular extracts were obtained as previously described (Atlan *et al.*, 1989). (ii) *Cell wall and cytoplasmic extracts.* Proteins were extracted from the bacterial cell wall of *L. helveticus* using a modification of the procedure previously developed to release APII from *Lactobacillus delbrueckii* subsp. *bulgaricus* (Atlan *et al.*, 1989). After 4 h growth in 100 ml MRS or milk medium ($A_{600} = 0.8$ – 1.0), cells were harvested by centrifugation (10000 g, 10 min, 6 °C), washed in 25 mM-KH₂PO₄, 10 mM-MgCl₂ (pH 5.8) and resuspended in 10 ml of the same buffer supplemented with 0.6 M-sucrose and treated with 0.1 or 1 mg ml⁻¹ (for cells grown in MRS or milk medium, respectively) of lysozyme from chicken egg white (Sigma) for 30 min at 25 or 37 °C (for cells grown in MRS or milk medium, respectively) with gentle shaking. Bacterial suspensions were centrifuged (10000 g, 10 min, 6 °C) and the supernatants are referred to as lysozyme fluids. Lysozyme-treated bacteria were osmotically shocked by suspending them in 10 ml cold distilled water and maintained on ice for 10 min. Shocked cells were centrifuged (10000 g, 10 min, 6 °C) and these supernatants are referred to as osmotic fluids. The bacterial pellets were resuspended in 10 ml cold distilled water and disrupted with a French pressure cell (20000 p.s.i. [138 MPa], 6 °C; Amicon). The supernatants obtained after centrifugation of the broken cells (25000 g, 20 min, 6 °C) are referred to as soluble cytoplasmic fluids.

Enzyme assays. AP activities were assayed as previously described (Atlan *et al.*, 1989) with lysyl- (3 mM), arginyl- (10 mM) or leucyl-p-nitroanilide (pNa) (15 mM) as substrates. Alanine-proline-pNA (6 mM) was used as the substrate for assaying pro-dipeptidyl aminopeptidase activity (Pro-DPAP). One unit (U) of AP or Pro-DPAP activity was defined as the amount of enzyme that hydrolysed 1 μ mol substrate min⁻¹.

Casainolytic activity was determined as previously described (Atlan *et al.*, 1990). β -Galactosidase activity was assayed with *o*-nitrophenyl- β -D-galactopyranoside (3 mM) as the substrate (Miller, 1972). One unit of enzyme activity was defined as the amount of enzyme that hydrolysed 1 nmol substrate min⁻¹.

Hydrolysis of various peptides was detected by TLC. APII activity was assayed as follows. The reaction mixture, containing 2 mM-substrate in 20 mM-HEPES (pH 7.0), and an appropriate amount of enzyme, was incubated for 60 min at 40 °C. The reaction mixture (10 μ l) was then spotted onto a precoated silica gel 60 plate with a gel layer thickness of 0.25 cm (Merck). A 4:1:1 (by vol.) mixture of *n*-butanol/acetic acid/water was used as the mobile phase. As a control, 2 mM of each standard peptide and amino acid were also spotted onto the plate. Silica gels were stained by spraying them with 0.1% ninhydrin in ethanol. Peptides and amino acids became visible after the silica gel was incubated for 5 min at 80 °C.

Electrophoretic analysis. SDS-PAGE was done by the method of Laemmli (1970). Polyacrylamide gel gradients (7–15%, w/v) were routinely used. Before electrophoresis, samples were boiled for 3 min in 0.1 M- β -mercaptoethanol in the presence of 1% (w/v) SDS. Electrophoresis continued for 13 h at a constant 16 mA. After migration, gels were stained at 50 °C with 0.1% Coomassie brilliant blue in methanol/acetic acid/water (50:10:40, by vol.) and then diffusion destained for 12 h at 30 °C in successive baths of methanol/acetic acid/water (5:10:85, by vol.).

The purified APII fraction was analysed by PAGE in 7% (w/v) acrylamide gel without SDS. Migration was in 100 mM-HEPES buffer, pH 7.4, at 12 °C. Enzyme activity was detected directly on the gel by incubation in the following mixture: 10 mg Arg- β -naphthylamide in 1 ml dimethylformamide and 50 mg Fast Garnet GBC (Sigma) in 30 ml 50 mM-H₂NaPO₄/HNa₂PO₄ buffer, pH 7.0. Hydrolysis of the substrate was correlated with the appearance of a red colour at the level of the enzyme.

Purification of aminopeptidase II. Osmotic fluids (100 ml) were prepared from *L. helveticus* ITGL1 grown in milk medium using the procedure described above (1 mg lysozyme ml⁻¹ for 30 min at 37 °C) and were lyophilized. Freeze-dried extracts were resuspended in 10 ml 20 mM-Tris/HCl, pH 7.5. The following purification steps were carried out at 4 °C. (i) *First ion-exchange chromatography.* The concentrated extract was applied to a column of Mono-Q HR 5/5 (Pharmacia) equilibrated with 20 mM-Tris/HCl, 7.5. A linear gradient of NaCl (0–0.5 M) in the same buffer was run over 10 min at a flow rate of 1 ml min⁻¹ and 0.5 ml fractions were collected. Fractions hydrolysing lysyl-pNA with the highest activity were pooled and concentrated with an Amicon filtration unit using a XM-10 membrane (molecular mass cutoff 10 kDa; Amicon).

(ii) *Gel filtration chromatography.* The concentrated active extract was applied to a column of Superose 12 HR 10/30 (Pharmacia) and eluted at a flow rate of 0.5 ml min⁻¹ with 20 mM-Tris/HCl, pH 7.5 containing 40 mM-NaCl. Fractions with the highest lysyl-AP activity were pooled and concentrated by filtration on a XM10 membrane in 20 mM-Tris/HCl buffer, pH 7.5. The molecular mass of the active enzyme was estimated in these conditions, and the column was calibrated using Sigma standards: cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), BSA (66 kDa), alcohol dehydrogenase (150 kDa) and β -amylase (200 kDa).

(iii) *Second ion-exchange chromatography.* The second ion-exchange chromatography was done as described for the first step, but with a linear NaCl gradient of 0.15–0.28 M in 26 min at a flow rate of 0.5 ml min⁻¹.

Heat treatment. Enzyme extracts were treated at 62 °C as previously described (Atlan *et al.*, 1989).

Isoelectric focusing. Isoelectric focusing in polyacrylamide gels was done as previously described (Laloi *et al.*, 1991) with ampholine pH 4–6 (LKB), 40 mM-glutamate as anolyte and 0.2 M-histidine as catholyte. The following proteins were used as pI markers: carbonic anhydrase A from human erythrocytes (Sigma, pI 6.57), carbonic anhydrase B from bovine erythrocytes (Boehringer, pI 5.85), β -lactoglobulin A from bovine milk (Sigma, pI 5.20) and trypsin inhibitor from soybean (Sigma, pI 4.55).

Protein determination. The protein content of enzyme preparations was determined by the method of Bradford (1976), using BSA as the standard.

Effect of divalent cations and chemical reagents on APII activity. The purified enzyme was preincubated in the presence or absence of various ions or inhibitors for 30 min at 4 °C. After addition of the substrate (lysyl-pNA), enzyme activity was determined at 50 °C in 20 mM-MES,

Table 1. Enzymic analysis of parental strain and AP-deficient mutants of *L. helveticus*

Total extracts were prepared from cells grown for 4 h in MRS or milk medium. Data are means of three independent cultures.

Growth medium and strain	Enzyme activity [U (mg bacterial dry wt) ⁻¹]					
	Lysyl-AP	Arginyl-AP	Leucyl-AP	X-Pro-DPAP	Caseinolytic	β -Galactosidase
MRS medium						
Parental strain ITGL1	24	22	12	30	1187	118
ITGL2	0.5	1.1	1.6	22	885	106
ITGL3	0.6	1.7	2.3	29	1163	100
Milk medium						
Parental strain ITGL1	28	26	17	37	10996	83
ITGL2	0.5	0.5	2.9	35	8054	76
ITGL3	0.25	0.4	4.1	48	11086	94

pH 6.5. EDTA- or 1,10-phenanthroline-treated enzyme was pre-incubated for 30 min at 4 °C with different ions in 20 mM-MES, pH 6.5 before adding the substrate.

Results and Discussion

Proteolytic activities in parental and AP-deficient strains of L. helveticus

Total extracts from *L. helveticus* parental strain ITGL1, grown either in MRS or in milk medium, contained approximately equal amounts of peptidase activities responsible for the hydrolysis of lysyl-, arginyl-, leucyl- or alanyl-proline-pNA, (Table 1). However, the caseinolytic activity of strain ITGL1 grown in milk was nine-fold higher than that of cells grown in MRS (Table 1). Similar results have been obtained with *Lactobacillus delbrueckii* subsp. *bulgaricus*, where the biosynthesis of the cell-surface proteinase depends on the nature of the culture medium (Laloi *et al.*, 1991).

Mutant strains ITGL2 and ITGL3 were derived from *L. helveticus* ITGL1 by mutagenesis with nitrosoguanidine and identified on the basis of their inability to hydrolyse leucyl- β -naphthylamide (Prost & Chamba, 1990) by using an *in situ* enzymic plate assay as described previously (Atlan *et al.*, 1989). These mutants showed a marked decrease in leucyl-AP activity (Table 1) as well as lysyl- and arginyl-AP activities. Strain ITGL2 displayed caseinolytic and X-Pro-DPAP activities ranging from 75 % to 100 % of parental activities. We noted that X-Pro-DPAP activity of strain ITGL3 was slightly enhanced (1.3-fold) after growth in milk medium. β -Galactosidase activity, used as an independent marker of the proteolytic system, was not altered in the mutants (Table 1).

Subcellular location and characterization of two APs in L. helveticus

No lysyl-, arginyl- or leucyl-AP activity was detected in the culture medium during growth of *L. helveticus* ITGL1 (data not shown). Bacteria were treated with increasing concentrations of lysozyme, then osmotically shocked to release cell-wall-associated proteins. No significant AP activity was detected in the lysozyme fluids obtained from the first step, therefore we only analysed the effects of temperature, treatment time and lysozyme concentration on the release of AP activities into the osmotic fluid (second step) (Table 2).

When cells were grown in milk medium the optimal temperature (37 °C) and lysozyme treatment (1 mg ml⁻¹ for 30 min) induced the release of about 55 % of the three AP activities (lysyl-, arginyl and leucyl-AP) into the osmotic fluid without significant cellular lysis (< 2.8 %) as estimated by assaying β -galactosidase activity, a cytoplasmic marker, and X-pro-DPAP activity, which were all unreleased by lysozyme treatment in *L. delbrueckii* subsp. *bulgaricus* (Atlan *et al.*, 1990). After growth in MRS medium, lysozyme concentration and treatment temperature were modified to minimize cellular fragility: in these conditions, 33 % of the three AP activities were released during osmotic shock at 25 °C in the presence of 0.1 mg lysozyme ml⁻¹.

The cellular location of leucyl-AP activity was analysed in the mutant strains ITGL2 and ITGL3 after growth in milk medium and treatment with 1 mg lysozyme ml⁻¹ for 30 min at 37 °C (Table 3). High amounts of residual leucyl-AP activity (65 and 76 % for ITGL2 and ITGL3, respectively) were released into osmotic fluids without significant cell lysis (1.8 and 4.5 %, respectively).

These results indicate that the enzymes responsible for lysyl-, arginyl- and leucyl-AP activities are localized in the cell wall. A similar method had previously allowed us

Table 2. *Effect of lysozyme concentration and temperature on the release of AP activities into osmotic fluids from L. helveticus parental strain grown in MRS or milk medium*

Treatment		Release of enzyme activity (%)*									
		β -Galactosidase		Lysyl-AP		Arginyl-AP		Leucyl-AP		X-Pro-DPAP	
		MRS	Milk	MRS	Milk	MRS	Milk	MRS	Milk	MRS	Milk
0.05	25	1.9	—	15.1	—	17	—	15.6	—	2.9	—
0.08	25	1.1	—	19.5	—	21.4	—	20.1	—	—	—
0.1	10	0.1	—	0.4	—	1.1	—	0	—	—	—
0.1	25	3.6	0.4	30.8	0.8	33	1	34.4	—	4	—
0.1	30	25.6	—	71.9	—	73.2	—	77.2	—	32.3	—
0.15	25	14.2	0.4	55.3	2.1	58.4	—	61	—	27.9	—
0.25	25	16.6	1	69.1	3.4	—	—	—	—	—	—
0.4	25	21.6	—	77.4	—	—	—	—	—	—	—
0.6	25	—	0.2	—	3	—	—	—	—	—	—
1	25	—	0.2	—	4.7	—	—	—	—	—	—
1	30	—	0.3	—	16	—	18.4	—	—	—	1.2
1	37	—	1	—	55	—	56.5	—	52	—	2.8
2	25	—	0.1	—	6.5	—	7.8	—	—	—	0.9

* 100 % Activity is defined as the sum of the activities measured in the three extracts (lysozyme, osmotic and cytoplasmic fluids).

—, Not determined.

Table 3. *Distribution of leucyl-AP activity in different cellular extracts from peptidase deficient mutants of L. helveticus*

Data are mean values from 3 independent cultures.

Strain and cellular fraction	Leucyl-AP		β -Galactosidase		X-Pro-DPAP	
	Activity*	(%)	Activity*	(%)	Activity*	(%)
ITGL2 mutant						
Lysozyme fluid	0.1	4	0.15	0.2	0.2	0.6
Osmotic fluid	1.7	65	0.45	0.6	0.6	1.8
Cytoplasmic fluid	0.8	31	74.3	99.2	33.1	97.6
Total	2.6	100	74.9	100	33.9	100
ITGL3 mutant						
Lysozyme fluid	0	0	0.26	0.3	0.3	0.7
Osmotic fluid	2.8	76	1.6	1.8	1.9	4.5
Cytoplasmic fluid	0.9	24	86.5	97.9	39.9	94.8
Total	3.7	100	88.4	100	42.1	100

* Activity is expressed in enzyme units (mg bacterial dry weight)⁻¹.

to differentially release APII, a specific enzyme responsible for lysyl-pNA hydrolysis, from *Lactobacillus delbrueckii* subsp. *bulgaricus* (Atlan *et al.*, 1989). Treatment of osmotic fluids from *L. helveticus* parental cells grown in milk medium with the metal chelating agents EDTA (0.1 mM) and 1,10-phenanthroline (1 mM) completely inhibited the lysyl- and arginyl-AP activities, but only 80 % of the leucyl-AP activity. Moreover, residual leucyl-AP activity of osmotic fluids from mutant strains ITGL2 and ITGL3 was stimulated by 0.1 mM-EDTA. Similar results were obtained for AP activities in osmotic fluids prepared from cells grown in MRS medium (data not shown). These results strongly suggest that at least

two different enzymes are involved in leucyl-AP activity.

Osmotic fluids prepared from parental cells grown in MRS or milk medium were mixed and heat-inactivated at 62 °C to determine if the same enzyme(s) is (are) synthesized during growth in both culture media. Residual lysyl-, arginyl- and leucyl-AP activities were measured as a function of time. The logarithm of lysyl- and arginyl-AP activities decreased linearly with inactivation time to about 5 % of the residual activities without a change in the slope and with a half-time of 7.5 min (Fig. 1). These results show that whatever culture medium was used, a single cell-wall-associated enzyme was responsible for the hydrolysis of lysyl- and arginyl-pNA. On the

Fig. 1. Heat inactivation kinetics of lysyl- or arginyl- (▲) and leucyl-AP (●) activities of *L. helveticus* ITGL1. Residual AP activities were estimated after heat treatment (62 °C) of a mixture (1:1, v/v) of osmotic fluids prepared from parental strain ITGL1 grown either in MRS or milk medium.

other hand, the kinetics of heat inactivation at 62 °C of leucyl-AP activity were biphasic (Fig. 1), which suggests that at least two APs are involved in the leucyl-AP activity located in the *L. helveticus* cell wall.

Taken together, these data prompt us to propose that a single metal-containing enzyme is responsible for lysyl- and arginyl-AP activities as well as a part of the leucyl-AP activity. We named this enzyme APII, in agreement with the nomenclature proposed by Ezzat and coworkers (Ezzat *et al.*, 1986). Moreover, a second AP contributes to leucyl-AP activity with properties similar to those of APIV from *Lactobacillus delbrueckii* subsp. *bulgaricus* (Atlan *et al.*, 1989); we therefore propose the same name, APIV, for this AP of *L. helveticus*. The properties of APII-deficient mutants confirmed the presence of APIV.

Cell wall protein composition of APII deficient mutants

The protein composition of cell wall extracts prepared from parental and mutant strains grown in milk medium (see Methods) was analysed by SDS-PAGE. No signifi-

Fig. 2. SDS-PAGE analysis of the proteins released into lysozyme and osmotic fluids from *L. helveticus* parental and AP-deficient strains grown in milk medium. Lane a, molecular mass markers: phosphorylase B (96 kDa), catalase (58 kDa), glutamic dehydrogenase (53 kDa), alkaline phosphatase (45 kDa); lanes b, c, d, lysozyme fluids from the parental strain and the mutants ITGL2 and ITGL3, respectively; lanes e, f, g, osmotic fluids from the parental strain, ITGL2 and ITGL3, respectively. Extract samples loaded on the gel were obtained from 5×10^8 cells.

cant difference was observed between the lysozyme fluids (Fig. 2, lanes b–d). The protein patterns of osmotic fluids, however, were more complex (Fig. 2, lanes e–g). Similar electrophoretic protein profiles were displayed by parental and ITGL2 mutant strains, whereas mutant ITGL3

Table 4. Purification of APII from *L. helveticus* ITGL1

Purification step	Total activity (mmol lysyl-pNA min ⁻¹)	Total protein (mg)	Specific activity [nmol lysyl-pNA (mg protein) ⁻¹ min ⁻¹]	Yield (% activity)	Purification (-fold)
Osmotic fluid	6.9	8	0.86	100	1.0
Anion exchange chromatography (Mono-Q HR 5/5)	4.4	0.41	10.5	63	12.2
Gel filtration (Superose 12 HR 10/30)	2.4	0.12	19.7	34.5	22.8
Anion exchange chromatography (Mono-Q HR 5/5)	0.73	0.03	24.2	10.5	28.0

Fig. 3. SDS-PAGE analysis of protein fractions during the purification of APII. Lanes: *a*, ITGL3 osmotic fluid (24 µg protein); *b*, *c*, *d* and *e*, ITGL1: *b*, ITGL1 osmotic fluid (24 µg protein); *c*, active pooled fractions after the first MonoQ chromatography (10 µg protein); *d*, active pooled fractions after gel filtration (2 µg protein); *e*, active pooled fractions after the second MonoQ chromatography (1.5 µg protein); *f*, standards: phosphorylase B (96 kDa), catalase (58 kDa), glutamic dehydrogenase (53 kDa), carbonic anhydrase (30 kDa) and lysozyme (14 kDa).

was characterized by a total deficiency of peptide A (96 kDa) and an increase in peptides B (90 kDa) and C (66 kDa) (Fig. 2, lane *g*). Similar results were obtained after cell growth in MRS medium (data not shown).

Purification of APII

Osmotic fluids prepared from *L. helveticus* ITGL1 cells grown in milk medium were used as enriched extracts for APII purification. The enzyme was purified by three steps of column chromatography as described in Methods. The results of a typical purification are summarized in Table 4. Step 1: During the first anion exchange chromatography, lysyl-pNA-hydrolysing activity eluted at 0.28 M-NaCl. In this fraction, 63 % of total activity, corresponding to 5 % of the total amount of protein, was recovered. Step 2: The concentrated active fraction was applied to a Superose 12 gel filtration

column. After elution, the specific activity of the lysyl-pNA-hydrolysing fraction was increased 1.9-fold. Step 3: The concentrated APII fraction was applied to a MonoQ anion exchange column; after chromatography, the purified enzyme eluted at 0.23 M-NaCl.

By this procedure, the enzyme was purified 28-fold from osmotic fluid extracts with a yield of 10.5 %.

Properties of APII

The molecular mass of APII was estimated to be 97 000 Da by HPLC with a Superose 12 column. SDS-PAGE of the purified enzyme fraction resulted in a single protein band corresponding to a molecular mass of 96 000 Da (Fig. 3, lane *e*). After non-denaturing PAGE, a single red band with the same relative mobility as the purified fraction was observed when enzyme activity was revealed by direct staining on the gel with L-lysyl-β-naphthylamide (data not shown). The molecular masses of the APs from *L. helveticus* CNRZ32 and LHE-511 are 97 and 92 kDa, respectively (Khalid & Marth, 1990*b*; Miyakawa *et al.*, 1992). The protein band for the APII of *L. helveticus* ITGL1 as identified after SDS-PAGE corresponded to polypeptide A, which was absent in the osmotic fluid prepared from mutant ITGL3. This strain may have a nonsense or a deletion mutation altering the APII structural gene or a mutation in a regulatory determinant. In mutant ITGL2, APII was synthesized but inactive; its structural gene might be altered by a missense mutation in an essential site for enzyme activity. The pI of the enzyme was estimated as 4.8 by IEF (data not shown).

Temperature and pH dependence of APII. The effect of temperature on APII activity was measured in the range of 5–65 °C. The purified enzyme was equilibrated in 20 mM-MES, pH 6.5 for 5 min at the appropriate temperature before adding lysyl-pNA. The optimum temperature for enzyme activity was 50 °C. At 55 °C, the enzyme still displayed 50 % of the activity measured at 50 °C. At 60 °C, no hydrolysing activity could be detected. APs from *L. helveticus* CNRZ32 and LHE-511 are most active at 45 °C and 37 °C, respectively (Khalid & Marth, 1990*b*; Miyakawa *et al.* 1992). On the other hand, AP from *L. helveticus* LHE-511 loses activity at temperatures above 47.5 °C. Therefore, the APII we purified is much more thermotolerant.

The effect of pH on enzyme activity was examined at 50 °C in the range of pH 4–10 by using a polybuffer composed of 20 mM each of malic acid, MES, HEPES and boric acid, adjusted to the appropriate pH values. The optimum pH for enzyme activity was 6.5. At pH values below 5 and above 9, no hydrolysing activity could be detected. The APs from *L. helveticus* CNRZ32 and LHE-511 are most active at pH 6.5 and 7.0,

Table 5. *Relative activity of purified APII on several p-nitroanilide derivatives*

The rate of hydrolysis of L-lysine-pNA was taken as 100%.

Substrate	Relative activity (%)
Lys-pNA	100
Arg-pNA	29
Leu-pNA	13
Ala-pNA	9
Pro-pNA	< 1
Ala-Pro-pNA	1.6
Gly-Pro-pNA	< 1
Val-Ala-pNA	< 1

Table 6. *Substrate specificity of purified APII*

Substrate	Hydrolysis	Substrate	Hydrolysis
Ala-Gly	—	Pro-Ala	—
Ala-Pro	—	Pro-Phe	—
Ala-Leu	++	Pro-Leu	—
Ala-Phe	++	Pro-Pro	—
Ala-His	++	Pro-Gly	—
Ala-Met	++	Pro-Hydroxypro	—
Gly-Val	—	Val-Leu	+
Gly-Trp	—	Val-Pro	—
Gly-Phe	—	Val-Gly	—
Gly-Tyr	—	Zgly-Leu	—
Gly-Ala	—	NCBZ-Gly-Phe	—
Gly-Pro	—	Val-Gly-Gly	—
His-Ser	—	Gly-Pro-Ala	—
Leu-Pro	—	Leu-Leu-Leu	++
Leu-Gly	—	Leu-Ala-Pro	++ in leu and ala-pro
Leu-Ala	++	Pro-Gly-Gly	—
Phe-Pro	—		
Lys-Tyr	—		

++ , Complete hydrolysis.

+ , Slight hydrolysis detectable.

— , No detectable hydrolysis.

respectively. However, they are active under acidic conditions (pH 5.0 and 4.5, respectively) (Khalid & Marth, 1990b; Miyakawa *et al.*, 1992).

Substrate specificity of APII. Table 5 shows the relative rates of hydrolysis for various substrates with C-terminal pNA. The highest activities were found with lysyl-pNA and to a lesser extent with arginyl- and leucyl-pNA. APs from *L. helveticus* CNRZ32 and LHE-511 exhibit similar properties (Khalid & Marth, 1990b; Miyakawa *et al.*, 1992). The kinetic parameters of APII were determined using lysyl-pNA as the substrate. The enzyme hydrolysed lysyl-pNA with a K_m of 0.29 mM and a V_{max} of 100 mmol min⁻¹ (mg protein)⁻¹ (data not shown).

The hydrolytic action of APII towards various peptides was analysed by TLC (Table 6). APII showed a marked preference for di- and tripeptides with Leu

or/and Ala. Peptides containing proline or glycine were not hydrolysed. In contrast, AP from *L. helveticus* CNRZ32 hydrolysed the gly-tyr and gly-phe dipeptides (Khalid & Marth, 1990b). No carboxypeptidase activity was detected with the substrate NCBZ-Gly-Phe.

Effect of various chemical reagents and metal ions on APII activity. Enzyme activity was measured in the presence of different classes of inhibitors or metal ions to obtain some information about the active site of APII. Inhibitors of metalloenzymes such as EDTA (0.1 mM) and 1,10-phenanthroline (1 mM), totally inhibited the purified enzyme. On the other hand, inhibitors of serine enzymes (PMSF), compounds acting on the SH groups of proteins [monoiodoacetic acid 1 mM, E64 (*N*-[*N*-(L-3 transcarboxyoxirane-2-carbonyl)-L-leucyl]-agmatine; Boehringer-Mannheim) 1 mM] and reducing agents (dithiothreitol, 1 mM, β -mercaptoethanol, 1 mM) had no effect on APII activity. These results suggested that intact disulphide or thiol groups are not essential for enzyme activity. In contrast, AP from *L. helveticus* CNRZ32 is inhibited by thiol-blocking reagents such as *p*-chloromercuribenzoate, *N*-ethylmaleimide, iodoacetate and iodoacetamide, indicating that thiol groups are essential for this enzyme.

The activity of EDTA-treated APII was totally recovered by the addition of 1 mM-CoCl₂, but only 44 % or 26 %, respectively, was restored by the addition 1 mM-CaCl₂ or 1 mM-MnCl₂. Addition of 1 mM-CoCl₂ restored 35 % of the activity of 1,10-phenanthroline-treated APII. The enzyme was totally inhibited by 1 mM-ZnCl₂ or 1 mM-CuCl₂, and partially inhibited by MnCl₂ at 0.1 mM (54 %) or 1 mM (90 %) or CoCl₂ at 1 mM (17 %) or 10 mM (73 %). High concentrations (10 mM) of CaCl₂ and MgCl₂ had no effect on enzyme activity. AP activities from *L. helveticus* CNRZ32 and LHE-511 are strongly activated by Co²⁺ (1 mM) and partially inhibited (10 % and 30 %, respectively) by Zn²⁺ (1 mM) (Khalid & Marth, 1990b; Miyakawa *et al.* 1992).

Conclusions

The development of a differential extraction procedure for cell envelope proteins of *L. helveticus* allowed us to show that two components of the proteolytic system, APII and APIV, were localized in the cell wall. We have shown that these enzymes displayed different properties: APII is a metallo-enzyme with a broad substrate specificity, and is the only AP from *L. helveticus* able to hydrolyse lysyl-pNA, whereas APIV is responsible for a part of the leucyl-AP activity but is not a metallo-enzyme.

APII showed similar properties (molecular mass, optimum pH) with *L. helveticus* CNRZ32 and *L. helveticus* LHE-511 APs, but had no essential SH group,

unlike AP from strain CNRZ32. Moreover, compared to the LHE-511 AP, APII had a much higher optimal temperature.

The broad substrate specificity, cell wall location and several other properties of the purified enzyme suggest that APII from *L. helveticus* ITGL1 contributes to the degradation of casein. APII-deficient mutants (ITGL2 and ITGL3) have been used for Emmental cheese production, which resulted in flavour defects (Prost & Chamba, 1990). The bitter flavour which results from protein degradation is apparently caused by an accumulation of bitter peptides containing a high proportion of hydrophobic amino acids. The APII of *L. helveticus* ITGL1 might contribute to the development of cheese flavour by processing bitter peptides. Research is now in progress to purify APIV from osmotic fluids of ITGL3 mutant strain, devoid of APII.

We are grateful to Bernard Dequatre for excellent technical assistance. We thank the Institut Technique du Gruyère for providing aminopeptidase-deficient strains. This work was supported by grants from the Centre National de la Recherche Scientifique (UMR 106) and the Université Claude Bernard.

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