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# X-Prolyl-Dipeptidyl Aminopeptidase of *Lactobacillus delbrueckii* subsp. *bulgaricus*: Characterization of the Enzyme and Isolation of Deficient Mutants

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***Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397 is able to hydrolyze X-proline-*para*-nitroanilides and X-proline-β-naphthylamides (X for alanyl- or glycyl-). A single metal-independent cytoplasmic enzyme with a molecular weight estimated to be 82,000 is responsible for these activities and was named X-prolyl-dipeptidyl aminopeptidase (X-Pro-DPAP). Isolation and analysis of mutants totally deficient for X-Pro-DPAP activity showed that a total lack of this enzyme induces (i) a decrease in the growth rate; (ii) an increase in cell wall proteinase activity; (iii) the loss of three cell wall proteins with respective molecular masses of 16, 40, and 52 kilodaltons; and (iv) enhancement of a cell wall protein with a molecular mass of 150 kilodaltons. The involvement of X-Pro-DPAP in casein catabolism is discussed.**

To grow in milk, lactic acid bacteria have to synthesize a set of proteinases and exopeptidases able to hydrolyze and degrade milk proteins because their requirement in essential amino acids cannot be satisfied by the low concentration of free amino acids in the medium (14). The proteolytic system of *Lactobacillus delbrueckii* subsp. *bulgaricus* is complex; its analysis has started only very recently. Three proteinases localized at the cell surface have been partially characterized (8), and two intracellular dipeptidases have been purified (7). Previously, we have shown that *L. delbrueckii* subsp. *bulgaricus* CNRZ 397 synthesizes four aminopeptidases (AP I to IV); AP II is a metallo-enzyme specifically assayed with lysyl-*para*-nitroanilide (lysyl-pNA) as substrate and is localized in the cell wall (2). None of these AP were able to split peptide bonds involving the imino group of proline. Lactic acid bacteria should therefore contain specific endopeptidases and/or exopeptidases towards proline to break down casein-derived polypeptides (caseins are rich in this amino acid, corresponding to 11.3% of casein dry weight) (1, 14). *L. delbrueckii* subsp. *bulgaricus* is devoid of proline endopeptidase activity but contains a low level of proline iminopeptidase activity and a high level of X-prolyl-dipeptidyl AP activity (X-Pro-DPAP), which is responsible for the release of an X-Pro dipeptide when proline is at the penultimate position from the amino terminus (4). X-Pro-DPAP (EC 3.4.14) has been purified from several lactic acid bacteria: *Lactococcus lactis* subspp. *cremoris* and *lactis* and *Streptococcus thermophilus* (9, 11). No study was carried out on the X-Pro-DPAP of *L. delbrueckii* subsp. *bulgaricus*.

We have previously isolated and characterized mutants of *L. delbrueckii* subsp. *bulgaricus* CNRZ 397 deficient in AP II which displayed an increase in glycyl-Pro-DPAP activity after growth in milk medium (2). These results suggested that the biosynthesis of AP II and the enzyme(s) involved in the glycyl-Pro-DPAP activity might be coregulated. In this paper, we report the characterization of the X-Pro-DPAP and describe the isolation and analysis of mutants altered for this activity.

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## MATERIALS AND METHODS

**Bacteria and culture conditions.** *L. delbrueckii* subsp. *bulgaricus* CNRZ 397 was obtained from the Centre de Recherches I.N.R.A., Jouy-en-Josas, France. Stock cultures were maintained frozen at −30°C in 10% (wt/vol) skim milk. Cultures were grown at 40°C in MRS broth (Difco Laboratories, Detroit, Mich.) (6) or reconstituted 10% skim milk solution (Elle & Vire powder; Union Laitière Normande, Condé-sur-Vire, France) prepared as described previously (2). The composition of the synthetic medium has been published previously (2). Solid media contained 1.5% agar.

One unit of absorbance at 600 nm in a spectrophotometer (SP320; Jouan, Saint-Nazaire, France) corresponds to 250 μg of bacterial dry weight per ml (2). Proteins were assayed according to the method described by Schacterle and Pollack (13).

**Mutagenesis and identification of mutants.** *L. delbrueckii* subsp. *bulgaricus* CNRZ 397 was mutagenized with nitrosoguanidine (50 μg/ml, 60 min; Serva, Heidelberg, Federal Republic of Germany), as we already described (2). Under these conditions, there was 50% survival. After an overnight growth on milk agar, mutants were identified for their inability to hydrolyze glycyl-proline-β-naphthylamide by using an in situ enzymatic plate assay (2).

**Cell permeabilization.** Bacteria grown in 8 ml of MRS medium were centrifuged (25,000 × *g*, 10 min, 6°C), washed with 4 ml of 0.2 M Tris hydrochloride (pH 7.0), and treated with 0.2 ml of toluene-acetone (1:9, vol/vol) according to a published method (5).

**Cell fractions. (i) Total cellular extracts.** Bacterial cells grown in 100 ml of milk or MRS medium were harvested as previously described (2), washed with 0.2 M Tris hydrochloride (pH 7.0), disrupted with a French pressure cell (20,000 lb/in$^2$ at 6°C; Aminco, Urbana, Ill.) and spun down (25,000 × *g*, 20 min, 6°C) to remove unbroken cells and cell debris. Supernatants were called total cellular extracts.

**(ii) F1, F2, and F3 extracts.** Proteins were extracted from the bacterial cell wall according to a modified procedure previously developed to release AP II (2). Bacteria grown in milk or MRS medium were harvested in the mid-exponential

TABLE 1. Enzyme activities assayed in total cellular extracts from parental strain and Gly-Pro-DPAP mutants of *L. delbrueckii* subsp. *bulgaricus* CNRZ 397[a]

| Growth medium/enzyme activity | Enzyme activity (U/mg of bacterial dry weight) | | | | |
|---|---|---|---|---|---|
| | Parental strain | X-Pro-DPAP altered strains | | | |
| | | 1104 | 1105 | 1107 | 1106 |
| Growth in milk medium | | | | | |
| Gly-Pro-DPAP | 39.2 | 0 | 0 | 3.1 | 14.0 |
| Ala-Pro-DPAP | 88.0 | 0 | 0 | 8.0 | 60.0 |
| Lysyl-AP (AP II) | 25.6 | 27.2 | 26.0 | 28.4 | 21.6 |
| Arginyl-AP | 29.2 | 30.4 | 32.4 | 31.2 | 26.8 |
| Leucyl-AP | 6.8 | 5.6 | 6.8 | 5.2 | 25.2 |
| β-Galactosidase | 2,216 | 2,176 | 2,168 | 2,080 | 1,884 |
| Growth in MRS medium | | | | | |
| Gly-Pro-DPAP | 46.4 | 0 | 0 | 3.6 | 18.0 |
| Ala-Pro-DPAP | 98.0 | 0 | 0 | 5.7 | 56.8 |
| Lysyl-AP (AP II) | 21.6 | 23.6 | 20.4 | 22.4 | 22.8 |
| Arginyl-AP | 28.0 | 30.8 | 28.8 | 27.2 | 28.0 |
| Leucyl-AP | 6.4 | 7.6 | 6.4 | 7.2 | 5.6 |
| β-Galactosidase | 2,204 | 2,040 | 1,876 | 1,896 | 1,804 |

[a] Cells were grown during 4 h in MRS or milk medium. Data are mean values from three independent cultures.

phase of growth (optical density at 600 nm of 0.8 to 1.0), washed twice with 25 ml of 50 mM $KH_2PO_4$ (pH 7.0), resuspended in 10 ml of the same buffer, and treated with lysozyme (Sigma Chemical Co., St. Louis, Mo.; 1 mg/ml) for 30 min at 4°C under a slight shaking. Bacterial suspensions were spun down (10,000 × *g*, 10 min, 6°C), and the resulting supernatants were named F1 extracts. Lysozyme-treated bacteria were carefully suspended in 10 ml of cold distilled water and maintained for 10 min on ice. Afterwards, bacterial suspensions were centrifuged (10,000 × *g*, 10 min, 6°C) and supernatants were retained as F2 extracts; bacterial pellets were resuspended in 10 ml of cold distilled water, and cell disintegration was performed with a French pressure cell (20,000 lb/in$^2$ at 6°C). Supernatants obtained after centrifugation of broken cells (25,000 × *g*, 20 min, 6°C) were called F3 extracts.

**Enzyme assays.** AP II activity was determined by following the hydrolysis of lysyl-pNA; arginyl- and leucyl-AP activities were assayed with arginyl- or leucyl-pNA, respectively, as substrates. These methods were described by Atlan et al. (2). Alanyl-proline-pNA (6 mM) or glycyl-proline-pNA (5 mM) were used as substrates for assaying X-Pro-DPAP activity. One unit of AP or X-Pro-DPAP corresponded to the amount of enzyme that hydrolyzed 1 μmol of substrate per min.

Caseinolytic activity was determined by measuring the trichloroacetic acid-soluble fluorescence released from fluorescein isothiocyanate-labeled casein according to a modification of the procedure published by Twinning et al. (2, 15). One relative fluorescent unit corresponded to the fluorescence at 450 nm of a 48 μM quinine sulfate solution under an excitation wavelength of 350 nm. Each value was the mean of data from five independent assays.

A standard published procedure was used for assaying β-galactosidase activity with *o*-nitrophenyl-β-D-galactopyranoside as the substrate (12). One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 nmol of substrate per min.

**Heat treatment.** Enzyme extracts were treated at 55°C as previously described (2).

**Electrophoretic analysis.** Before electrophoresis, extracts were dialyzed against distilled water, concentrated by freeze-drying, and boiled under reducing conditions as we previously described (3). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (10) with a Protean II slab cell system (Bio-Rad Laboratories Richmond, Calif.). Routinely, sodium dodecyl sulfate-polyacrylamide gels (7 to 15% acrylamide gradient) were run overnight at 10°C and 13 mA constant current. Proteins were stained with 0.1% Coomassie brilliant blue R250 (Prolabo, Paris, France) in methanol–acetic acid–water (50:10:40, vol/vol/vol) for 1.5 h at 50°C. Gel destaining was carried out in successive washings with methanol–acetic acid–water (5:10:85, vol/vol/vol). After the boiling treatment, the following proteins were used as molecular weight markers: phosphorylase *b* from rabbit muscle (96,000), catalase from bovine liver (58,000), glutamic dehydrogenase from beef liver (53,000), carbonic anhydrase from bovine erythrocytes (30,000), and lysozyme from chicken egg white (14,000).

**Partial purification and gel chromatography.** Streptomycin sulfate was added up to 55% saturation into 20 ml of a total cellular extract prepared from *L. delbrueckii* subsp. *bulgaricus* CNRZ 397 grown in MRS medium and containing 74 mg of protein and 315 U of X-Pro-DPAP activity. After 2 h at 4°C, the precipitated nucleic acids were removed by centrifugation (20,000 × *g*, 30 min, 4°C). Solid ammonium sulfate was added to the supernatant up to 75% saturation, and the suspension was maintained overnight at 4°C. Precipitated proteins were recovered by centrifugation (20,000 × *g*, 30 min, 4°C) and suspended into 1.0 ml of 50 mM Tris hydrochloride buffer, pH 7.0. This partially purified extract containing 98% of the initial X-Pro-DPAP activity was loaded on a column (1.6 by 70 cm) of Ultrogel AcA44 (IBF, Villeneuve-la-Garenne, France). Chromatography was carried out as we described previously (2).

## RESULTS

**Characterization of X-pro-DPAP.** *L. delbrueckii* subsp. *bulgaricus* parental strain CNRZ 397 grown in milk or MRS medium contained approximately equal amounts of enzyme activities associated with the hydrolysis of Gly-Pro- and Ala-Pro-pNAs (Table 1). In agreement with previously published results (2), lysyl-, arginyl- and leucyl-AP activities were also independent of growth conditions (Table 1).

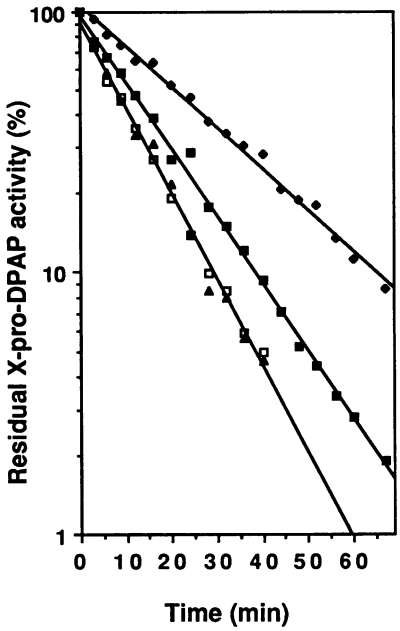


FIG. 1. Heat inactivation kinetics of X-Pro-DPAP. Total cellular extracts were treated at 55°C as described previously (2). (i) Residual Ala-Pro- (□) and Gly-Pro-DPAP (▲) activities were measured in a total cellular extract prepared from an MRS culture of parental strain CNRZ 397 containing 202 U of Ala-Pro- and 144 U of Gly-Pro-DPAP activity per ml. (ii) Gly-Pro-DPAP activity (◆) was estimated in a mixture (1/1, vol/vol) of total cellular extracts obtained from the parental strain grown in MRS and milk media containing 132 or 147 U/ml, respectively. (iii) Ala-Pro-DPAP activity (■) was assayed in a mixture (1:1, vol/vol) of total cellular extracts prepared from milk cultures of the parental strain and AP II-deficient strain 1000 containing 200 and 225 U/ml, respectively.

A total extract prepared from strain CNRZ 397 grown in MRS medium was heat inactivated at 55°C, and residual Gly-Pro- and Ala-Pro-DPAP activities were measured as a function of time (Fig. 1). The logarithm of these activities decreased linearly with inactivation time up to about 5% of the residual activities without a change in the slope and with a half-life of 10 min. These results indicated that whatever substrate was used, a single enzyme is responsible for Gly-Pro- and Ala-Pro-DPAP activities. We named it X-Pro-DPAP in agreement with the nomenclature used by other authors (9, 11). When total extracts from cells developed in MRS or milk medium, each of which contained equal amounts of Gly-Pro-DPAP activity, were mixed and inactivated at 55°C, linear kinetics were also observed (Fig. 1). These results strongly suggest that only one enzyme was synthesized, whatever the nature of the growth medium. Moreover, we checked that a single enzyme was responsible for Ala-Pro-DPAP activity from cells grown in milk medium (data not shown).

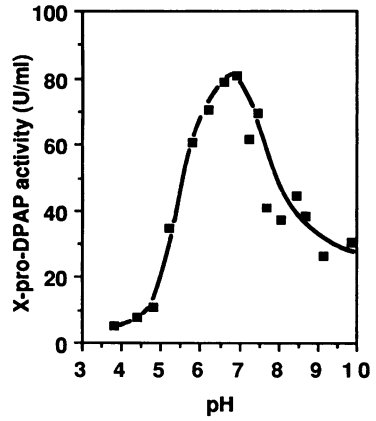


FIG. 2. Effect of pH on X-Pro-DPAP activity (pH 3.6 to 5.2, $CH_3COOH$-NaOH; pH 5.8 to 7.0, $KH_2PO_4$-$K_2HPO_4$; pH 7.2 to 9.9, Tris hydrochloride).

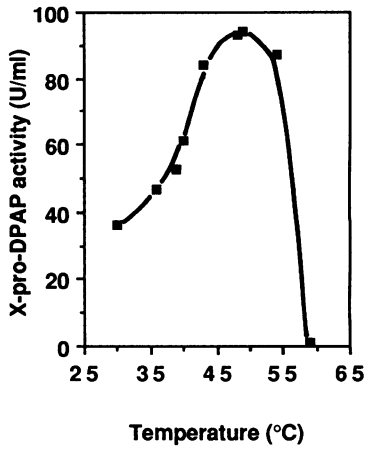


FIG. 3. Effect of temperature on X-Pro-DPAP activity.

X-Pro-DPAP activity was optimal for pH values close to 7.0 and a temperature of 50°C (Fig. 2 and 3, respectively). Apparent $K_m$ values for Ala-Pro- and Gly-Pro-pNAs were equal to 0.065 and 0.110 mM, respectively. The effects of various inhibitors on enzyme activity were determined (Table 2). X-Pro-DPAP activity was unaffected by the addition of $Co^{2+}$ (1 mM) or metal complexing reagents such as EDTA (10 mM) or *o*-phenanthroline (1 mM) but was severely inhibited by phenylmethylsulfonyl fluoride (10 mM) and $Cu^{2+}$ (1 mM). The molecular weight of X-Pro-DPAP was estimated to be 82,000 by gel filtration of a partially purified extract (see Materials and Methods).

**Biosynthesis of X-Pro-DPAP.** The kinetics of X-Pro-DPAP biosynthesis were followed during growth of the parental strain in MRS medium (Fig. 4). During the exponential-growth phase and after a 2-h lag period, enzyme activity linearly increased as a function of bacterial biomass. X-Pro-DPAP activity assayed on 24-h-old cultures and in the

TABLE 2. Effect of cations and proteinase inhibitors on the X-Pro-DPAP activity of *L. delbrueckii* subsp. *bulgaricus* CNRZ 397[a]

| Cations or inhibitors | X-Pro-DPAP activity (%) |
|---|---|
| No addition | 100 |
| $Co^{2+}$ (1 mM) | 98 |
| $Cu^{2+}$ (1 mM) | 43 |
| EDTA (1 mM) | 92 |
| EDTA (10 mM) | 82 |
| *o*-Phenanthroline (1 mM) | 94 |
| Phenylmethylsulfonyl fluoride (1 mM) | 46 |
| Phenylmethylsulfonyl fluoride (10 mM) | 20 |

[a] Enzyme activity was assayed on a total cellular extract with Ala-Pro-pNA as described in Materials and Methods.

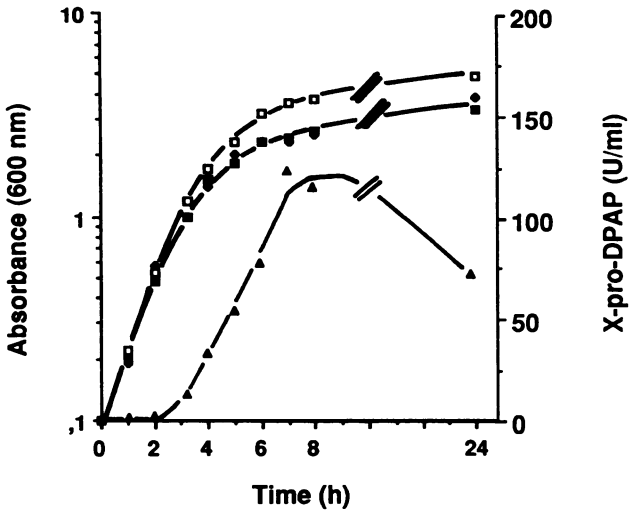


FIG. 4. Growth kinetics of parental strain CNRZ 397 (□) and X-Pro-DPAP-deficient mutants 1104 (■) and 1105 (◆) and biosynthesis kinetics of X-Pro-DPAP activity (▲) in parental strain. Cultures were carried out in MRS medium, and cells were permeabilized as described in Materials and Methods.

absence of cell lysis was lower than expected, suggesting that the enzyme was degraded (or inactivated) in stationary phase cells.

We have shown previously that the X-Pro-DPAP activity of AP II-deficient mutants grown in milk medium was significantly higher than that of parental strain (2). When total cellular extracts from parental and AP II-deficient strains developed in milk medium were mixed (each of which contained equal amounts of Ala-Pro-DPAP activity) and heat inactivated at 55°C, linear kinetics were obtained (Fig. 1). Moreover, when the apparent $K_m$ values for Ala-Pro-pNA were determined with extracts obtained from parental or AP II-deficient strains, similar values were obtained. Taken together, these results showed that the increase in X-Pro-DPAP activity observed with AP II mutants was the direct consequence of an increase in X-Pro-DPAP biosynthesis.

**Cell localization of X-Pro-DPAP.** X-Pro-DPAP activity was neither released into the culture medium during growth nor detected with whole cells of *L. delbrueckii* subsp. *bulgaricus* CNRZ 397. In order to release cell wall proteins, bacterial cells were treated according to a two-step procedure we have developed (see Materials and Methods). F1 extracts contained proteins released from the cell wall after a lysozyme treatment of bacterial cells, whereas the F2 extracts contained proteins ejected from lysozyme-treated cells by osmotic shock. Bacterial cells did not exhibit any lysis during these treatments, as estimated by measuring cytoplasmic β-galactosidase activity in F1 and F2 extracts. No significant amount of X-Pro-DPAP activity was released into F1 and F2 cell wall extracts, whereas 96% of this activity (Table 3) and 99% of β-galactosidase activity were recovered into the F3 fractions. On the other hand, when bacterial cells were first treated with lysozyme and mutanolysin and then subjected to an osmotic shock, they released, for most part, AP II activity (2) but a very low level of X-Pro-DPAP activity (data not shown). After centrifugation (258,000 × *g*, 1 h) of crude F3 fluids (containing membrane particles), 100% of the X-Pro-DPAP activity was recovered in the supernatant. These results suggested that X-Pro-DPAP is located in the cytoplasm.

TABLE 3. Distribution of X-Pro-DPAP and casein lytic activities in different cellular extracts from parental strain and X-Pro-DPAP-deficient mutants[a]

| Strain/cellular fraction | Enzyme activity from MRS culture | | | | Enzyme activity from milk culture | | | |
|---|---|---|---|---|---|---|---|---|
| | X-Pro-DPAP[b] | | Caseinolytic | | X-Pro-DPAP | | Caseinolytic | |
| | (U) | (%) | (U) | (%) | (U) | (%) | (U) | (%) |
| Parental strain | | | | | | | | |
| F1 | 0 | 0 | 80 | 2 | 0 | 0 | 1,200 | 7 |
| F2 | 2.6 | 3 | 1,200 | 30 | 3.4 | 4 | 7,560 | 48 |
| F3 | 93.4 | 97 | 2,760 | 68 | 82.6 | 96 | 7,080 | 45 |
| Total[c] | 96.0 | 100 | 4,040 | 100 | 86.0 | 100 | 15,840 | 100 |
| 1104 | | | | | | | | |
| F1 | ND[d] | ND | 200 | 3 | ND | ND | 3,400 | 16 |
| F2 | ND | ND | 3,760 | 52 | ND | ND | 11,080 | 53 |
| F3 | 0 | ND | 3,200 | 45 | 0 | ND | 6,600 | 31 |
| Total[c] | 0 | ND | 7,160 | 100 | 0 | ND | 21,080 | 100 |
| 1105 | | | | | | | | |
| F1 | ND | ND | 440 | 6 | ND | ND | 3,680 | 15 |
| F2 | ND | ND | 4,400 | 57 | ND | ND | 12,480 | 53 |
| F3 | 0 | ND | 2,800 | 37 | 0 | ND | 7,520 | 32 |
| Total[c] | 0 | ND | 7,640 | 100 | 0 | ND | 23,680 | 100 |

[a] Bacteria were grown during 4 h in MRS or milk medium. F1, F2, and F3 fractions were obtained as described in Materials and Methods.
[b] X-Pro-DPAP activity was measured with Ala-Pro-pNA as the substrate.
[c] Total activity was the sum of the activities measured in the three extracts (F1 to F3).
[d] ND, Not determined.

**Isolation and phenotypic characterization of mutants altered for X-Pro-DPAP.** The parental strain was mutagenized as described in Materials and Methods. After growth on milk agar, we used an in situ enzymatic assay to identify four independent clones (strains 1104 to 1107) altered for Gly-Pro-β-naphtylamide hydrolysis with a mutation frequency of $2 \times 10^{-4}$. Except for X-Pro derivatives, these mutants were able to hydrolyze all amino-acyl- and dipeptidyl-β-nahthylamides hydrolyzed by the parental strain (2).

Growth of X-Pro-DPAP-deficient mutants on solid synthetic medium required the same 13 amino acids as those required by parental strain CNRZ 397. Like the parental strain, mutants catabolized lactose, glucose, mannose, and fructose.

The growth curves of parental and totally X-Pro-DPAP-deficient strains (1104 and 1105) developing in MRS medium are given in Fig. 4. Both types of strains had a doubling time of 40 min; mutants were characterized by a shorter exponential phase and produced a significantly lower biomass than that of the parent (0.85 to 1.0 mg instead of 1.23 mg of bacterial dry weight per ml after 24 h of growth).

**Properties of X-Pro-DPAP-deficient mutants.** Mutant strains 1104 and 1105 did not contain any detectable X-Pro-DPAP activity, whereas strains 1107 and 1106 displayed X-Pro-DPAP activities ranging from 8 to 68% of parental activities (Table 1). All mutants synthesized as much AP and β-galactosidase activities as the parental strain; β-galactosidase was used as an enzyme marker independent of the proteolytic system. Similar results were obtained after bacteria were grown in milk or MRS medium.

**Synthesis and cell localization of caseinolytic activity in X-Pro-DPAP-deficient strains.** Total caseinolytic activities from parental and mutant strains developed in milk medium were three times higher than those measured with cells grown in MRS medium (Table 3). Whatever the growth conditions, mutant activities were reproducibly 1.5-fold

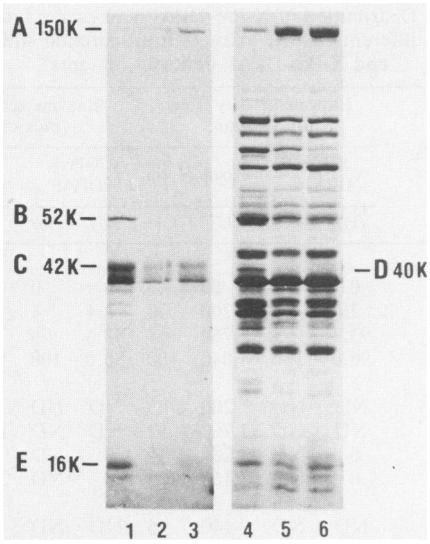


FIG. 5. Sodium dodecyl sulfate-polyacrylamide (7 to 15% gradient) gel electrophoretic analysis of proteins released into F1 and F2 fluids from parental strain (1, 4), mutant 1104 (2, 5), and mutant 1105 (3, 6), respectively. Strains were grown in milk medium during 4 h, and samples obtained from $5 \times 10^8$ cells were loaded on the gel.

higher than parental activities (Table 3). Furthermore, results from cell fractionation studies showed that the increase in enzyme activity observed with mutants was specifically associated with F1 and F2 cell wall extracts (Table 3). Whatever strain or culture medium used, 99% of total β-galactosidase activity was recovered in F3 extracts, which shows that no significant lysis did occur during cell treatments.

**Effect of X-Pro-DPAP deficiency on protein composition of the cell wall.** The protein composition of cell wall extracts prepared from parental or mutant strains grown in milk medium was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Figure 5 shows that a dozen polypeptides were detected in F1 fluid from the parental strain; peptide C, with an apparent molecular mass of 42 kilodaltons (kDa), was specifically released into F1 extracts. Protein patterns of F2 extracts were more complex. Mutants were characterized (i) by an increase of peptide A (150 kDa) in F1 and F2 fluids, (ii) a total deficiency of peptides B (52 kDa) and E (16 kDa) in F1 fluids, (iii) and a significant decrease of peptide D (40 kDa) in F2 fluids.

Moreover, the amounts of peptide A detected in F1 and F2 fluids obtained from parental and mutant strains developed in MRS medium (data not shown) were significantly lower than those detected in fluids from milk-grown cultures.

## DISCUSSION

Heat inactivation studies of Ala-Pro- and Gly-Pro-DPAP activities of *L. delbrueckii* subsp. *bulgaricus* CNRZ 397 as well as the analysis of mutants deficient in these activities led us to characterize an exopeptidase, X-Pro-DPAP, not previously described in this strain. This enzyme is specifically responsible for the hydrolysis of Ala-Pro- and Gly-Pro-pNAs, although it is much more active towards Ala-Pro-pNA as substrate. We have shown that X-Pro-DPAP is sensitive to $Cu^{2+}$ ions and phenylmethylsulfonyl fluoride, probably indicating a serine-protease type enzyme. This exopeptidase is characterized by a very sharp optimum pH (7.0) and a high optimal temperature (50°C). Its molecular mass was estimated to be 82 kDa by gel filtration of a total cellular extract. These biochemical properties are similar to those of X-Pro-DPAP recently purified from *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, except for a molecular mass which is equal to 160 to 180 kDa for the above bacteria (9, 11).

We have shown that X-Pro-DPAP of *L. delbrueckii* subsp. *bulgaricus* was not located in the cell wall, as was reported for *L. lactis* subsp. *cremoris* (9). In the latter bacteria, the authors suggested that X-Pro-DPAP is involved in the degradation of β-casein proline sequences because of the pattern of degradation of β-casomorphin (9).

Protein composition of the lactic acid bacteria cell envelope had never been analyzed. The development of a new differential extraction procedure allowed us to reveal that the cell wall of *L. delbrueckii* subsp. *bulgaricus* contains a great variety of proteins. Mutations responsible for a deficiency in X-Pro-DPAP induced a pleiotropic effect on the cell wall protein composition (see Fig. 5): the amount of three major peptides (B, D, and E) was significantly decreased, whereas the level of peptide A was markedly enhanced. Peptide A, whose concentration increased concomitantly with caseinolytic activity in milk-grown cells or in X-Pro-DPAP mutants, might be a proteinase component. However, it should be noted that the increase in peptide A in mutants was greater than the 1.5-fold increase in caseinolytic activity.

Our investigation on peptidase-deficient mutants has extended our knowledge of the proteolytic system of *L. delbrueckii* subsp. *bulgaricus*. We have shown that a deficiency in X-Pro-DPAP gave rise to a specific increase of cell wall proteinase activity. Similarly, AP II-deficient mutants grown in milk medium displayed an increase in cell wall proteinase and X-Pro-DPAP activities (2). These results strongly suggest the existence of common regulatory mechanisms controlling the biosynthesis of AP II, X-Pro-DPAP, and cell wall proteinases. These enzymes, which have different modes of action, bond specificities, and cellular localizations, might be involved in the casein degradation pathway providing essential amino acids to the bacterial cell. For a more precise study of the regulation of the proteolytic system, X-Pro-DPAP and AP II double mutants as well as peptidase-overproducing mutants have been isolated and are currently being analyzed. Moreover, further work in our laboratory is aimed at showing that single X-Pro-DPAP deficient mutants carry mutations in the structural gene of the enzyme.

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