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Enzymatic Ability of *Bifidobacterium animalis* subsp. *lactis* To Hydrolyze Milk Proteins: Identification and Characterization of Endopeptidase O

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The proteolytic system of *Bifidobacterium animalis* subsp. *lactis* was analyzed, and an intracellular endopeptidase (PepO) was identified and characterized. This work reports the first complete cloning, purification, and characterization of a proteolytic enzyme in *Bifidobacterium* spp. Aminopeptidase activities (general aminopeptidases, proline iminopeptidase, X-prolyl dipeptidylaminopeptidase) found in cell extracts of *B. animalis* subsp. *lactis* were higher for cells that had been grown in a milk-based medium than for those grown in MRS. A high specific proline iminopeptidase activity was observed in *B. animalis* subsp. *lactis*. Whole cells and cell wall-bound protein fractions showed no caseinolytic activity; however, the combined action of intracellular proteolytic enzymes could hydrolyze casein fractions rapidly. The endopeptidase activity of *B. animalis* subsp. *lactis* was examined in more detail, and the gene encoding an endopeptidase O in *B. animalis* subsp. *lactis* was cloned and overexpressed in *Escherichia coli*. The deduced amino acid sequence for *B. animalis* subsp. *lactis* PepO indicated that it is a member of the M13 peptidase family of zinc metallopeptidases and displays 67.4% sequence homology with the predicted PepO protein from *Bifidobacterium longum*. The recombinant enzyme was shown to be a 74-kDa monomer. Activity of *B. animalis* subsp. *lactis* PepO was found with oligopeptide substrates of at least 5 amino acid residues, such as met-enkephalin, and with larger substrates, such as the 23-amino-acid peptide α_{s1}-casein(f1-23). The predominant peptide bond cleaved by *B. animalis* subsp. *lactis* PepO was on the N-terminal side of phenylalanine residues. The enzyme also showed a post-proline secondary cleavage site.

Bifidobacteria are gram-positive anaerobic bacteria commonly found in the human intestinal tracts of mammals. Many bifidobacterium-containing dairy products have been developed due to their reported health-promoting effects. These organisms are employed to increase the beneficial properties of fermented milks, infant formulas, cheese, and ice cream (10, 28, 31, 43). One of the strains commonly used in the industry is *Bifidobacterium animalis* subsp. *lactis*, which is particularly suitable due to its technological properties such as tolerance to oxygen, acid resistance, and ability to grow in milk-based media (20, 30, 33).

Information in the literature regarding the metabolism of bifidobacteria focuses mainly on their glycolytic capabilities, since these organisms have been reported to grow well on oligosaccharide-based substrates (21, 49). Analysis of the genome sequence of *Bifidobacterium longum* NCC2705 revealed a large number of predicted proteins specialized for oligosaccharide metabolism (45). Genetic and biochemical characterization of *Bifidobacterium* glycosyl hydrolases has identified several enzymes that utilize nondigestible oligosaccharides as substrates (19, 21, 22, 26). On the other hand, very little is known about the proteolytic enzyme systems of *Bifidobacterium* spp. However, analysis of the *B. longum* NCC2705 genome predicted more than 20 peptidases, including general aminopeptidases, peptidases specific for proline-residues, dipeptidases, and endopeptidases, as well as ABC-type transporter systems specific for oligopeptides. No genes encoding proteins similar to PrtP, a cell envelope-associated proteinase, were identified in the NCC2705 genome.

Endopeptidases are of particular interest due to their ability to hydrolyze peptide bonds within an oligopeptide. Several endopeptidases have been identified and characterized in *Lactobacillus* spp., *Streptococcus thermophilus*, and *Lactococcus lactis* (5–8, 13, 37); all of these are metalloproteases. Although their physiological role is unclear, the implication of these enzymes in the degradation of peptides produced during cheese manufacturing and ripening, such as α_{s1}-casein(f1-23), has been described (1, 7, 8). In previous studies, we have reported that the poor growth of *B. animalis* subsp. *lactis* in milk could be enhanced by supplementation of the medium with whey peptide fractions (glycomacropeptide [GMP] and whey protein concentrate) (20, 28). Therefore, the present study has focused on the proteolytic ability of *B. animalis* subsp. *lactis* to utilize milk proteins and milk-derived peptides. Special attention was given to the endopeptidase PepO, which was cloned, overexpressed, and characterized, particularly for its specificity against α_{s1}-casein(f1-23).

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Escherichia coli* strains XL1-Blue (4) and BL21(DE3)pLysS (Novagen, Madison, Wis.) were used as cloning and expression hosts, respectively. Cells were routinely grown at 37°C in Luria-Bertani medium (44) supplemented with 12.5 μg/ml tetracycline, 30 μg/ml kanamycin, 34 μg/ml chloramphenicol, and/or 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) when appropriate. *Bifidobacterium animalis* subsp. *lactis* DSM 10140^T was cultured in MRS broth (Pronadisa, Madrid, Spain) and a milk-based

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TABLE 1. Chromogenic substrates and corresponding references to the methods employed for the determination of proteolytic activities in *Bifidobacterium animalis* subsp. *lactis*

Activity	Substrate	Reference
Proteinase	Azocasein	46
	Universal Protease Substrate	42
	Succinyl-Ala-Glu-Pro-Phe-*p*-nitroanilide	29
Endopeptidase	*N*-Glutaryl-Ala-Ala-Phe-4-methoxy-β-naphthylamide	25
	N-Benzoyl-Val-Gly-Arg-*p*-nitroanilide	6
	N-Benzoyl-Pro-Phe-Arg-*p*-nitroanilide	
	N-Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide	
X-prolyl-dipeptidyl aminopeptidase	Arg-Pro-*p*-nitroanilide	11
Proline iminopeptidase	Pro-*p*-nitroanilide	11
General aminopeptidases	Leu-*p*-nitroanilide	11
	Lys-*p*-nitroanilide	

medium, which contained 100 g/liter reconstituted skim milk powder (Scharlau, Barcelona, Spain) and 10 g/liter GMP (Lacprodan; Arla Food Ingredients, Denmark). Cells grown in this milk-based medium were propagated first in MRS broth and then in skim milk enriched with 5 g/liter yeast extract and 5 g/liter glucose. All media were supplemented with 0.5 g/liter L-cysteine hydrochloride to lower the redox potential. *B. animalis* subsp. *lactis* cultures were incubated at 37°C under anaerobic conditions (Gas-Pack, Anaerogen; Oxoid, Basingstoke, United Kingdom) for 24 h.

Cell fractionation. Cells were collected from MRS broth (1×10^9 CFU/ml) and milk-based medium (4×10^8 CFU/ml) by centrifugation (10,000 × *g*, 10 min, 4°C). Before centrifugation, the milk culture was neutralized at pH 6.5 with 1 M NaOH and cleared by adding 1% trisodium citrate to cause casein micelle dispersion, as previously described (14). Whole cells were washed with 2 mM Ca^{2+}-containing 50 mM Tris buffer, pH 8, and taken to an optical density at 600 nm (OD_{600}) of 20. The cell wall-bound fraction (CWF) was obtained by incubating the cell pellet obtained from milk cultures at 25°C for 1 h in 20 mM Bis-Tris buffer, pH 6.0, containing 10 mM EDTA as described by Fernández de Palencia et al. (16). The protein material of the fraction was precipitated with 80% ammonium sulfate and resuspended in 20 mM sodium phosphate buffer, pH 7, after centrifugation (20,000 × *g*, 5 min, 4°C). Cell extracts (CFE) were obtained from MRS and milk *B. animalis* subsp. *lactis* culture pellets using mechanical disruption by mixing cells (1:1) with glass beads (diameter, 150 to 212 μm; Sigma, St. Louis, Mo.) and vortexing the ice-cooled suspensions four times over 4 min. Cell debris and glass beads were collected by centrifugation (12,000 × *g*, 5 min, 4°C). Protein concentrations were determined as described by Bradford (3) using a commercial reagent (Bio-Rad Laboratories, Hercules, Calif.) with bovine serum albumin as a standard.

Screening for proteolytic activity of *B. animalis* subsp. *lactis* with chromogenic substrates. Whole cells, CFE, and CWFs were tested for proteolytic activity with chromogenic substrates. Proteinase activity was examined in whole cells (OD_{600}, 20) and CWFs (30 mg/ml). Endopeptidase, X-prolyl-dipeptidyl aminopeptidase, and aminopeptidase activities were determined in CFE (2 mg/ml). The substrates employed (all purchased from Sigma, except for succinyl-Ala-Glu-Pro-Phe-*p*-nitroanilide [Bachem, Bubendorf, Switzerland] and Universal Protease Substrate [Roche Diagnostics, Mannheim, Germany]) and references for the corresponding methods are listed in Table 1. All enzymatic reactions were performed in 50 mM sodium phosphate buffer, pH 7, at 37°C. Results are expressed as specific activity (units per milligram of protein). One unit of enzyme activity is defined as the amount of enzyme required to release 1 μmol chromogenic substrate per min under the enzyme assay conditions.

Analysis of galactosidase activity. The α- and β-galactosidase activities were measured by employing *o*-nitrophenyl-α-D-galactopyranoside and *o*-nitrophenyl-β-D-galactopyranoside (Sigma), respectively. The reaction mixture contained 50 mM sodium phosphate buffer, pH 6.5, 1 mM magnesium chloride, 1 mM 2-mercaptoethanol, 2.32 mM substrate, and CFE (2 mg/ml). The liberation of *o*-nitrophenol was observed at 410 nm and 37°C, and activity is expressed as units per milligram of protein. One unit is defined as the amount of enzyme that can release 1 μmol of *o*-nitrophenol per min under the given conditions.

Hydrolysis of milk proteins and casein-derived peptides by *B. animalis* subsp. *lactis*. Activities against caseins, whey proteins, GMP, and α_{s1}-casein(f1-23) were studied with whole cells, CWFs, CFE, and purified recombinant PepO. Whole cells were employed in the reaction mixture at a final OD_{600} of 20, CFE at a final concentration of 2 mg/ml protein, CWFs at 30 mg/ml, and recombinant PepO at 10 μg/ml. Pure α-, β-, and κ-caseins (Sigma) were suspended in 50 mM sodium phosphate buffer, pH 7, and used at a final concentration of 1 mg/ml, while α-lactoalbumin and β-lactoglobulin (Sigma) were used at a final concentration of 0.5 mg/ml. Aliquots were taken from the reaction mixtures after 2, 4, and 24 h of incubation and centrifuged (12,000 × *g*, 5 min), and the supernatants were mixed 1:1 with solubilization buffer (125 mM Tris buffer, pH 7.5, 20% glycerol, 4% sodium dodecyl sulfate [SDS], 0.01% bromophenol blue, and 10% mercaptoethanol). Samples were denatured (100°C, 5 min), and the hydrolysis products were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 15% polyacrylamide gels as described by Laemmli (24). Activities against GMP (Sigma) and α_{s1}-casein(f1-23) (a gift from P. Fernández de Palencia [15]) were assayed in 50 mM sodium phosphate buffer, pH 6, with 1 mg/ml and 4 mg/ml substrate, respectively. After a 50-min reaction, samples were centrifuged (12,000 × *g*, 5 min), and the supernatants were heat inactivated (100°C, 5 min). Hydrolysis products were analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) following the procedures described by Exterkate et al. (12) for α_{s1}-casein(f1-23) and by Martín-Diana et al. (27) for GMP. Peptides generated by enzymatic hydrolysis were collected and freeze-dried, and the N-terminal amino acid sequence was determined by automated Edman degradation analysis at the Protein Synthesis Laboratory in the Centro de Investigaciones Biológicas (CSIC, Madrid, Spain).

DNA manipulations. *B. animalis* subsp. *lactis* chromosomal DNA was obtained as described by Meile et al. (34). *E. coli* plasmid isolation was done with the GFX Micro Plasmid Prep kit (Amersham Biosciences, Uppsala, Sweden). DNA manipulations and electrotransformation of *E. coli* cells were carried out by standard methods (44).

Cloning and expression of the endopeptidase gene (*pepO*). *B. animalis* subsp. *lactis pepO* was identified by sequence similarity within a fragment of chromosomal *B. animalis* subsp. *lactis* DNA obtained and sequenced in the course of a random genome sampling of *B. animalis* subsp. *lactis* (unpublished results). To express the predicted *B. animalis* subsp. *lactis* endopeptidase gene in *E. coli*, a 2.28-kb DNA fragment was amplified by PCR using *B. animalis* subsp. *lactis* chromosomal DNA as a template and the primer pair NcoI_for (GAAGCCATGGATACCACTTTGCA ATCAGGC)–HindIII_rev (TATCAAGCTTCCAGATGCGCACGCGTTC) (underlined sequences indicate NcoI and HindIII restriction sites, respectively). DNA amplification was performed with *Pwo* DNA polymerase (Roche) according to the manufacturer's instructions. The NcoI/HindIII-digested PCR product was inserted into the Novagen pET-28a(+) vector. The recombinant plasmid, pET-*pepO*, encoding the complete endopeptidase gene as well as a C-terminal His_6 tag, was transformed into *E. coli* BL21(DE3)pLysS. The entire *pepO* sequence was confirmed by DNA sequencing.

Expression of the *B. animalis* subsp. *lactis pepO* gene was induced by adding IPTG (final concentration, 1 mM) to *E. coli* BL21(DE3)pLysS cells containing pET-*pepO* and growing them to an OD_{600} of approximately 0.5. For optimal expression, 0.8 mM glucose was also added to the culture medium. After a further 4 h of incubation, the cells were harvested by centrifugation (10,000 × *g*, 10 min). The production and the cellular localization of the target protein were verified by analysis of total proteins, soluble and insoluble cytoplasmic fractions, and the medium fraction according to the pET System Manual (39). IPTG-induced cultures of *E. coli* BL21(DE3)pLysS harboring pET-28a(+) were used as a negative control. The protein pattern of cell fractions was monitored by SDS-PAGE (24).

Purification of recombinant PepO. All steps for recombinant protein purification were carried out at 4°C. The induced cell pellet was frozen at −80°C, resuspended in 2 ml/g (wet weight) of binding buffer (0.02 M sodium phosphate, pH 7.4, 0.5 M NaCl, 0.01 M imidazole), and then mixed (1:1, wt/vol) with glass beads to obtain CFE. These CFE were used to purify the recombinant PepO by immobilized metal affinity chromatography (IMAC), employing a 1-ml HiTrap Chelating HP column (Amersham) according to the manufacturer's instructions. The enzyme was eluted with 0.1 M imidazole, and the solution was desalted (PD-10 column; Amersham) and stored in 0.02 M sodium phosphate buffer, pH 6, at −80°C. The molecular mass and purity of the recombinant enzyme were confirmed by native PAGE on 7.5% polyacrylamide gels using a nondenatured protein molecular weight marker (Sigma) as a standard.

TABLE 2. Aminopeptidase and galactosidase activities in CFE of *Bifidobacterium animalis* subsp. *lactis* cells cultured in MRS or milk supplemented with 10 g/liter GMP

Substrate	Mean sp act (U/mg)[a] ± SD in CFE from the following medium:	
	MRS	Milk + GMP
Arg-Pro-*p*-nitroanilide	ND[b]	0.057 ± 0.004
Pro-*p*-nitroanilide	ND	0.129 ± 0.002
Leu-*p*-nitroanilide	ND	0.060 ± 0.004
Lys-*p*-nitroanilide	ND	0.026 ± 0.002
o-Nitrophenyl-α-D-galactopyranoside	0.005 ± 0.000	0.350 ± 0.018
o-Nitrophenyl-β-D-galactopyranoside	0.019 ± 0.001	2.330 ± 0.086

[a] One unit is defined as the amount of enzyme required to release 1 μmol of *p*-nitroaniline or *o*-nitrophenol per min.

[b] ND, not detected; activity was lower than the lowest quantifiable level (0.005 U/mg).

Effects of reagents, pH, and temperature. All enzymatic reactions were carried out with bradykinin as the substrate. The purified recombinant PepO was preincubated with various metals and reagents at a 1 mM concentration in 50 mM sodium phosphate buffer, pH 6. The temperature optimum was determined by performing the enzymatic reactions at different temperatures (5 to 55°C), and the pH optimum was measured over pH ranges from 3 to 9 by employing different buffers, namely, sodium acetate (pH 3 to 5), sodium phosphate (pH 6 to 7), and Tris-HCl (pH 8 to 9). Thermostability was analyzed by incubating the enzyme at temperatures between 5 and 55°C and measuring the residual activity under standard conditions. pH stability was determined by incubating the enzyme at different pH values (pH 3 to 9) before measuring residual activity under standard conditions.

Substrate specificity and identification of hydrolysis products. The activity of the recombinant enzyme was measured toward bradykinin, angiotensin, substance P, methyl enkephalin, and methyl enkephalinamide (Sigma) as peptide substrates. Standard enzyme assays in 100 μl were carried out in 50 mM sodium phosphate buffer, pH 6, containing 4 μg/ml recombinant PepO and 0.16 mM peptide. After incubation for 50 min at 37°C, the reaction was stopped with 50 μl 3% trifluoroacetic acid, and the hydrolysis products were analyzed by RP-HPLC as described by Pritchard et al. (40). Hydrolysis of caseins, whey proteins, GMP, and α_{s1}-casein(f1-23) by the recombinant enzyme was also analyzed as described above. Hydrolysis products were collected, freeze-dried, and identified by amino acid sequencing.

Nucleotide sequence accession number. The entire *pepO* sequence was confirmed by DNA sequencing and deposited in the GenBank and EMBL databases (accession no. AJ844608).

RESULTS

Screening for proteolytic activity of *B. animalis* subsp. *lactis* with chromogenic substrates. Proteinase activity was not detected in CWFs or in whole cells with chromogenic substrates. The same negative result was obtained for endopeptidase activity measured with chromogenic substrates in CFE. However, X-prolyl-dipeptidyl aminopeptidase and general aminopeptidase activities could be detected in CFE (Table 2). CFE obtained from cells grown in MRS medium showed no detectable activity, whereas the same experiments performed with CFE obtained from cells grown in milk-based medium showed measurable activities, among which proline iminopeptidase activity was remarkably high. Analysis of galactosidase activities in *B. animalis* subsp. *lactis* CFE (Table 2) showed 100-fold-higher α- and β-galactosidase activities in cells grown in milk than in cells grown in MRS. The results indicate that the activities in CFE from MRS-grown cells were below the lowest quantifiable level (0.005 U/mg).

Hydrolysis of milk proteins and casein-derived peptides by *B. animalis* subsp. *lactis*. Whole cells, CWFs, and CFE of *B. animalis* subsp. *lactis* grown in milk-based medium were incubated with pure α-, β-, and κ-caseins to analyze caseinolytic activity. CFE could hydrolyze casein fractions completely after 4 h of incubation at 37°C as observed by SDS-PAGE analysis. Both β- and κ-caseins were hydrolyzed more rapidly than α-casein (results not shown). CWFs could not degrade caseins even after 24 h of incubation. Whole cells started to degrade caseins only after a 24-h incubation, which could be a result of cell lysis and leakage of intracellular material into the medium. To assess whether the cells were damaged, the corresponding supernatants were analyzed for β-galactosidase activity. The results showed that β-galactosidase activity was detected in the supernatant only in the 24-h-incubated samples. Whey proteins (α-lactoalbumin and β-lactoglobulin) could not be hydrolyzed by any of the cellular fractions analyzed.

Cloning and expression of the endopeptidase gene (*pepO*) in *E. coli* and purification of the recombinant protein. The endopeptidase gene of *B. animalis* subsp. *lactis* was identified by sequence similarity to the putative *B. longum* NCC2705 *pepO* (accession no. NC_004307) in the course of a random sampling of the genome of *B. animalis* subsp. *lactis*. The predicted *B. animalis* subsp. *lactis* endopeptidase gene (deposited under accession no. AJ844608) encodes a 693-amino-acid protein with a calculated molecular mass of 78.3 kDa, an isoelectric point of 4.6, and no predicted signal peptide or transmembrane domains, suggesting an intracellular location of the enzyme. The protein shared 67.4% amino acid sequence identity with the predicted endopeptidase of *B. longum* NCC2705 (accession no. NP_696878). A high degree of similarity was also found with predicted metalloendopeptidases from *Propionibacterium acnes* KPA171202 (YP_056588) (45.7% amino acid identity) and *Corynebacterium glutamicum* ATCC 13032 (NP_599406) (43.8% amino acid identity). However, the *B. animalis* subsp. *lactis* gene displayed relatively low identity with PepO enzymes

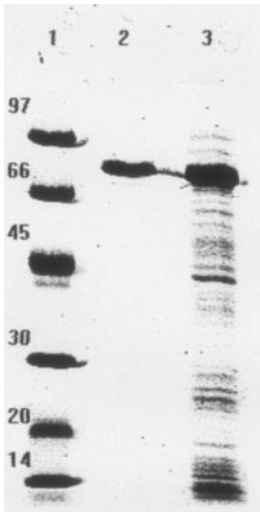

FIG. 1. SDS-PAGE analysis of purified protein fraction of IPTG-induced *E. coli* BL21(DE3)pLysS harboring the recombinant PepO. Lanes: 1, protein standard; 2, soluble cytoplasmic fraction eluted by IMAC with 0.1 M imidazole; 3, cell extract of 0.1 mM IPTG-induced *E. coli* BL21(DE3)pLysSpET-*pepO* cells.

TABLE 3. Effects of inhibitors and metal ions (1 mM) on the activity of recombinant PepO of *Bifidobacterium animalis* subsp. *lactis*

Substance	Mean residual activity[a] (±SD)
Phosphoramidon	23.46 (±5.92)
Diethyl pyrocarbonate	50.77 (±5.29)
Thiorphan	80.77 (±8.16)
EDTA	5.77 (±6.53)
1,10-Phenanthroline	0.00 (±0.86)
p-(Hydroxymercuri)benzoic acid	43.85 (±5.77)
PMSF	30.58 (±2.45)
Dithiothreitol	110.12 (±15.37)
Zn^{2+}	15.77 (±1.33)
Mn^{2+}	115.77 (±5.45)
Cu^{2+}	5.77 (±3.26)

[a] Expressed as a percentage of the activity on bradykinin in the absence of metal ions and chemical reagents, taken as 100% (0.064 U/mg).

TABLE 4. Substrate specificity and cleavage sites of recombinant PepO of *Bifidobacterium animalis* subsp. *lactis*

Substrate	Relative activity[a]	Cleavage sites[b]
Substrate P	100.0 (±0.0)	R-P-^{2}K-P-Q-Q-^{1}F-F-G-L-M-NH_2
Angiotensin	93.8 (±3.3)	D-R-V-Y-^{2}I-H-P-^{1}F-H-L
Bradykinin	38.8 (±7.8)	R-P-^{2}P-G-^{2}F-S-P-^{1}F-R
Met-enkephalinamide	10.5 (±2.7)	Y-G-G-^{1}F-M-NH_2
Met-enkephalin	10.2 (±4.8)	Y-G-G-^{1}F-M
α_{s1}-Casein(f1-23)	2.2 (±0.06)	R-P-K-H-P-I-K-H-Q-G-L-P-Q-^{1}E-V-L-N-E-N-L-L-R-F

[a] Expressed as a percentage of the activity toward substance P (1.7 U/mg), taken as 100%. Values are means (±standard deviations).
[b] ^{1}, primary cleavage site; ^{2}, secondary cleavage site.

from lactic acid bacteria (LAB), namely, 31.0%, 29.4%, and 28.5% identity with the PepO enzymes of *S. thermophilus* A (CAC14579), *Lactobacillus helveticus* CNRZ32 (O52071), and *Lactococcus lactis* subsp. *lactis* (F53290), respectively. The PepO-like endopeptidase of *B. animalis* subsp. *lactis* belongs to the M13 peptidase family of zinc metallopeptidases, since it contains all the catalytic signatures that are common to all the enzymes of this family, namely, ^{502}HExxH^{506}, ^{574}ExxxD^{578}, and ^{460}vNAfY^{464} (indices correspond to residue numbering in the *B. animalis* subsp. *lactis* PepO amino acid sequence).

Overexpression of the *pepO* gene in *E. coli* BL21(DE3)-pLysS occurred only when glucose was added to the growth medium along with IPTG. SDS-PAGE protein patterns of cellular fractions obtained from the IPTG-induced culture of *E. coli* BL21(DE3)pLysS cells bearing pET-*pepO* showed a strong signal at a molecular mass of about 74 kDa within the total protein and the soluble cytoplasmic fractions (Fig. 1). This size is in agreement with the calculated molecular mass (78 kDa) deduced from the predicted PepO sequence. The medium and insoluble cytoplasmic fractions contained only marginal amounts of protein, suggesting that most of the recombinant enzyme was available in a soluble form and thus probably in an active state (results not shown). Analysis of induced cells bearing pET-28a(+) showed no signal at 74 kDa within any fraction, confirming that the strong bands identified above originate from the expression of the cloned gene. As shown in Fig. 1, the C-terminal His_6 tag facilitated the purification to homogeneity of the recombinant PepO by a single IMAC step. Native PAGE of the recombinant protein showed one band with an apparent molecular mass of 74 kDa (results not shown), indicating that *B. animalis* subsp. *lactis* PepO is a monomeric enzyme.

Biochemical characterization of the recombinant *B. animalis* subsp. *lactis* PepO. For biochemical characterization, *B. animalis* subsp. *lactis* recombinant PepO activity toward bradykinin was measured. Optimal temperature and pH were 37°C and 6.5, respectively (results not shown). Residual activity decreased below 35% at pH values lower than 6.5, and no activity was measured below pH 5, while at pH 9 the residual activity was 48% (results not shown). The PepO was significantly inhibited by EDTA and 1,10-phenanthroline (Table 3), both of which are metalloenzyme inhibitors, but was only partially inhibited by phosphoramidon, an agent known to completely inhibit other metalloendopeptidases (48). Thiorphan, another specific inhibitor of endopeptidases, was even less effective at the inhibition of *B. animalis* subsp. *lactis* PepO than phosphoramidon (Table 3). Diethyl pyrocarbonate, which inhibits activity by reactivity with the active-site histidine (25), and the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) also caused partial inhibition. Dithiothreitol was the only agent that acted as an activator (Table 3), which could be due to its reducing character, whereas *p*-(hydroxymercuri)benzoic acid, which has an oxidizing effect, reduced the activity.

Since the enzyme was found to have no activity with synthetic chromogenic substrates, the hydrolysis of natural peptides was assessed (Table 4). Relative activities toward the different substrates were determined by following substrate disappearance as a function of time using RP-HPLC. In addition, the hydrolysis products were identified in order to determine the cleavage bond specificity. The predominant peptide bond cleaved by *B. animalis* subsp. *lactis* PepO was on the N-terminal side of phenylalanine residues. Main secondary cleavage sites were also at Pro-Xxx bonds. The specific activity of the enzyme was calculated on the basis of the primary cleavage site and before secondary products were released. Regardless of the preferential cleavage bonds, specific activity increased with peptide length, indicating that the enzyme functions as an endopeptidase (Table 4).

With respect to degradation of milk proteins and derived peptides, the pure recombinant PepO was not able to hydrolyze either α-, β-, or κ-casein, α-lactoalbumin, β-lactoglobulin, or GMP. However, α_{s1}-casein(f1-23), a 23-amino-acid peptide that is present in cheese whey as a consequence of enzymatic hydrolysis of α_{s1}-casein, was rapidly hydrolyzed by the recombinant enzyme. Hydrolysis of α_{s1}-casein(f1-23) was recorded during 20-, 40-, and 60-min incubations with PepO at 37°C (Fig. 2). The preferential cleavage site of PepO in α_{s1}-casein (f1-23) (Table 4) released peptide 3 (f14-23) and peptides 1 and 2, both of which started with the amino-terminal residues of α_{s1}-casein(f1-23). After 1 h of incubation with the recombinant PepO, little or none of the initial α_{s1}-casein(f1-23) could be detected, and the peptides originated underwent no further hydrolysis by this enzyme.

DISCUSSION

This study was conducted to characterize the proteolytic system of *B. animalis* subsp. *lactis* with special attention to the

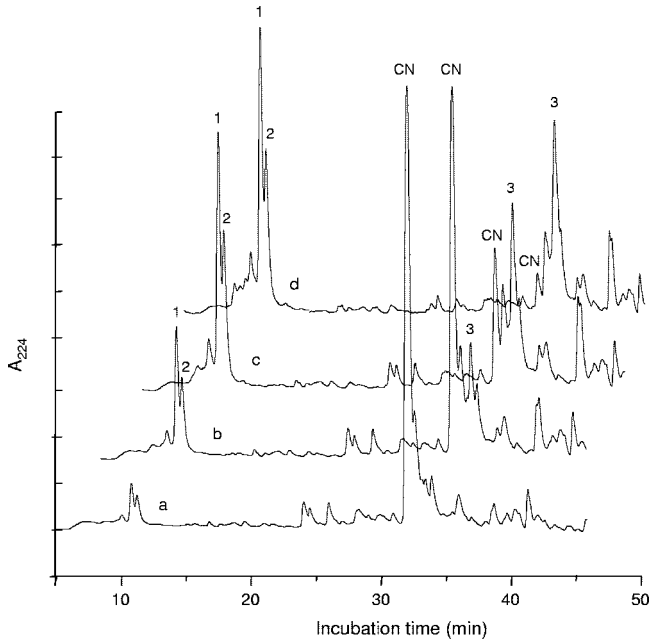

FIG. 2. Reverse-phase HPLC patterns of (a) pure α_{s1}-casein(f1-23) [CN] and its hydrolysis products (peaks 1, 2, and 3) after (b) 20-min, (c) 40-min, and (d) 60-min incubations with *Bifidobacterium animalis* subsp. *lactis* recombinant PepO.

identification and characterization of an intracellular endopeptidase. For the first time, we report the cloning, purification, and characterization of a proteolytic enzyme from a *Bifidobacterium* sp.

To gain insight into the proteolytic system of *B. animalis* subsp. *lactis*, cells were grown in a peptide-rich medium (MRS) or a milk-based medium with a low peptide content. By using chromogenic substrates, proteolytic activity was found only for intracellular aminopeptidases (Table 2) and the highest activity was measured in cells that had been grown in the milk-based medium. The increase in proteolytic activities observed when *B. animalis* subsp. *lactis* was grown in a milk-based medium is similar to reported findings for LAB, whose proteolytic activities are generally lower in cells grown in a peptide-rich medium, such as M17 or MRS, than in cells grown in milk (18, 23, 32). Interestingly, the specific proline iminopeptidase activity found in *B. animalis* subsp. *lactis* intracellular fractions is remarkably high (Table 2). This high activity with a substrate that contains an N-terminal proline residue is a distinctive characteristic of *B. animalis* subsp. *lactis* compared with LAB, since most LAB intracellular aminopeptidases have been reported to have a greater affinity for substrates containing lysine or leucine residues at the N-terminal position than for those containing proline (23, 41). Caseins are proteins rich in proline, and specialized peptidases are required to hydrolyze peptide bonds involving this imino acid (9).

No proteinase or endopeptidase activities were detected with specific chromogenic substrates in *B. animalis* subsp. *lactis* whole cells grown in MRS or milk-based medium or with their respective CFE. Therefore, these activities were assayed using milk proteins: caseins and whey proteins. Neither whole cells grown in milk nor CWFs were able to hydrolyze these proteins, indicating the absence of an extracellular proteinase activity under these conditions. However, the combined action of intracellular proteolytic enzymes could degrade caseins. Since some of the intracellular endopeptidases (PepO) identified in LAB have been reported to hydrolyze peptides derived from α_{s1}- and β-caseins (1, 7, 8), this enzyme was chosen for further characterization in *B. animalis* subsp. *lactis*.

The deduced amino acid sequence of *B. animalis* subsp. *lactis* PepO identified it as a member of the M13 family within the zinc metallopeptidase superfamily, whose members are also known as metzincins and whose main representatives are neprilysin (NEP), endothelin-converting enzyme 1 (ECE-1), and peptidase O (PepO). Phylogenetic analysis of *B. animalis* subsp. *lactis* PepO showed closer relationships with *Propionibacterium* and *Corynebacterium* PepO sequences than with LAB PepO sequences. This fact reflects the grouping of *Bifidobacterium* spp. as gram-positive bacteria with a high GC content in the class *Actinobacteria* (17). *B. animalis* subsp. *lactis* PepO contains the specific Zn^{2+}-binding domain and a conserved substrate binding and catalysis motif characteristic of these enzymes (2). In this sense, the enzyme was strongly inhibited by the divalent metal chelators EDTA and 1,10-phenanthroline. Specific inhibitors described for mammalian NEP and ECE-1, such as thiorphan and phosphoramidon (47), did not show a significant effect. The decrease in activity found with Zn^{2+} and PMSF (Table 3) has also been described for *L. lactis* PepO (47).

Activity of *B. animalis* subsp. *lactis* PepO was found toward substrates of at least 5 amino acid residues, such as met-enkephalin, as well as toward larger substrates, such as α_{s1}-casein(f1-23). However, the peptidase was not active with GMP, which is a 64-amino-acid-peptide. To a certain extent, enzyme specificity increased with peptide length (Table 4), as has been described for lactococcal PepO (25), but in contrast with mammalian NEP specificity. The enzyme has no proteinase activity.

The primary cleavage site of *B. animalis* subsp. *lactis* PepO (Table 4) is in agreement with the preferential cleavage site described for most LAB PepO enzymes (1, 5, 8, 25). However, *B. animalis* subsp. *lactis* PepO also showed a post-proline secondary cleavage site preference. The affinity of *B. animalis* subsp. *lactis* PepO for post-proline bonds and its inability to hydrolyze the chromogenic peptide substrates used to identify LAB PepO enzymes are also characteristics described for the PepO2 from *L. helveticus* (7).

Several duplicated endopeptidase activities have been described for *L. lactis* (PepO, PepF1, and PepF2 [35, 37, 38]) and for *L. helvetivus* (PepO, PepO2, and PepE [6, 7, 13]). However, the physiological role of PepO enzymes in LAB has not been clearly elucidated yet, since the growth of *L. lactis* or *L. helveticus pepO* deletion mutants in milk or chemically defined media was indistinguishable from that of the wild-type strains (6, 35, 36). The ability of *B. animalis* subsp. *lactis* PepO to hydrolyze α_{s1}-casein(f1-23) suggests that the enzyme may play a role in the increase in the growth of *B. animalis* subsp. *lactis* in milk when it is supplemented with whey peptide fractions (20, 28).

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