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Partial purification and characterization of an X-prolyl dipeptidyl aminopeptidase from *Lactobacillus sanfranciscensis* CB1

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Abstract

An X-prolyl dipeptidyl aminopeptidase (PepX) from *Lactobacillus sanfranciscensis* CB1, a key sourdough lactic acid bacterium, was partially purified by five chromatographic steps. As estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration, the enzyme appeared to be a 49.2–56 kDa monomer. Optimal activity occurred at pH 6.0 and 30 °C, with K_m of 0.6 mM and V_{max} of 99.6 nmol mg^{-1} min^{-1}. The *D* value, calculated at 45 °C, was ~5.46 s. The enzyme hydrolyzed (almost exclusively) substrates with a X-Pro N-terminal sequence. It did not possess prolidase, aminopeptidase or endopeptidase activities. No hydrolysis of the Pro-rich 33-mer epitope (a potent inducer of gut-derived human T-cell lines in celiac patients) was found when it was treated with PepX alone. When the general aminopeptidase type N was combined with PepX, the hydrolysis of 33-mer peptide (0.2 mM) was complete after 24 h of incubation at 30 °C. Leucine and glutamine residues were liberated from the Pro-rich 33-mer peptide by aminopeptidase type N, thus favouring the subsequent PepX activity. PepX was inactivated by *p*-chloromercuribenzoate, 3,4-dichloroisocoumarin and phenanthroline which exerted a competitive mode of inhibition. Among divalent cations, only Zn^{2+}, Hg^{2+} and Mn^{2+} markedly decreased the enzyme activity. Quadratic response surface methodology was used to study the individual and interactive effects of temperature, pH and NaCl on the PepX activity. The enzyme maintained considerable activity under the environmental conditions which characterize the sourdough fermentation.

Keywords: Lb. sanfranciscensis; Sourdough; X-prolyl dipeptidyl aminopeptidase

1. Introduction

Lactic acid bacteria are largely used in food fermentations. Beyond lactic acid fermentation, proteolysis by lactic acid bacteria is another important activity which may influence the flavour, texture and nutritional properties of several fermented foods (e.g., cheeses, fermented milks, sourdough breads and sausages). Lactic acid bacteria possess a complex proteolytic system, which consists of a cell wall-associated proteinase, transport systems specific for amino acids, di-, tri- and oligopeptides, and a number of intracellular peptidases (Kunji, Mierau, Hagting, Poolman, & Konings, 1996; Pritchard & Coolbear, 1993).

The X-prolyl dipeptidyl aminopeptidase, X-prolyl-dipeptidyl-peptidase or dipeptidyl peptidase IV (PepX, X-PDP or DPPIV) cleaves dipeptide residues from

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peptides or proteins by hydrolyzing the peptide bond at the carboxyl side of the proline residue when the imino acid is at the penultimate N-terminal position. PepX could be considered as the most studied proline-specific peptidase in dairy lactic acid bacteria since caseins are very rich in proline residues (Byun, Kofod, & Blinkovsky, 2001; Christensen, Dudley, Pederson, & Steel, 1999; Kunji et al., 1996). Proline is unique among the 20 amino acids because of its cyclic structure. This specific conformation imposes many restrictions on the structural aspects of peptides and proteins which may limit the extent of hydrolysis. Casey & Meyer (1985) first reported the presence of PepX in dairy lactic acid bacteria. Subsequently, PepX has been purified and characterized in several *Lactobacillus* species, such as *Lactobacillus curvatus* DPC2024 (Magboul & McSweeney, 2000), *Lactobacillus delbruecki* (Bockelmann, Fobker, & Teuber, 1991; Meyer & Jordi, 1987; Miyakawa, Kobayashi, Shimamura, & Tomita, 1991), *Lactobacillus acidophilus* (Bockelmann et al., 1991), *Lactobacillus casei* (Habibi-Najafi & Lee, 1994) and *Lactobacillus helveticus* (Miyakawa et al., 1994). PepX genes of *Lb. delbruecki* subsp. *bulgaricus* (Meyer-Barton, Klein, Imam, & Plapp, 1993), *Lb. helveticus* (Kimura, Nagasawa, Fujii, & Itoh, 2002; Vesanto, Savijoki, Rantanen, Steel, & Pavla, 1995) and *Lactobacillus rhamnosus* (Varmanen, Savijoki, Avall, Palva, & Tynkkynen, 2000) have been cloned and sequenced.

To our knowledge, PepX has not yet been described in sourdough lactobacilli. Gluten contains ~14.2% of proline residues (Carnovale, Marletta, & Ciuccio, 1995) and proteolysis during sourdough fermentation depends strictly on the activity of specific peptidases which hydrolyze peptide bonds where proline is located at the different positions as a potential substrate (Simpson, 2001). Overall, proteolysis during sourdough fermentation influences the growth of lactic acid bacteria and yeasts (Gobbetti, 1998), and free amino acids contribute directly, or as precursors, to bread flavour during sourdough fermentation and baking (Damiani et al., 1996). Therefore, if proteolysis proceeds slowly, or is stopped because of peptide bonds including proline, microbial growth and flavour development are negatively affected. Recently, it has been shown that proteolysis by sourdough lactobacilli may positively influence human tolerance to gluten (Di Cagno et al., 2004, 2002) due to the hydrolysis of Pro-rich gliadin oligopeptides which are responsible for the inappropriate T-cell mediated immune response of coeliac patients. The abundance and location of proline residues are crucial factors which contribute to the resistance of non tolerated peptides during breakdown by gastrointestinal enzymes (Shan et al., 2002). It has only been supposed that PepX may have a role in the degradation of Pro-rich oligopeptides which are responsible for coeliac disease (Hausch, Shan, Santiago, Gray, & Khosla, 2002).

The proteolytic system of *Lactobacillus sanfranciscensis* CB1, a key sourdough lactic acid bacterium, has been partially characterized: aminopeptidase type N, dipeptidase and cell-wall associated serine proteinase have previously been purified (Gobbetti, Smacchi, & Corsetti, 1996a). This paper describes the partial purification and biochemical characterization of an intracellular PepX of *Lb. sanfranciscensis* CB1.

2. Materials and methods

2.1. Reagents

DEAE cellulose was purchased from Sigma–Aldrich Ireland Ltd., Dublin, Ireland. *p*-Nitroanilide and AMC derivatives of amino acids and peptides were obtained from Bachem Feinchemikalien AG, Dubendorf, Switzerland and Sigma–Aldrich, respectively. Molecular weight standards for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and gel permeation chromatography were purchased from Sigma–Aldrich.

2.2. Sourdough lactic acid bacteria, culture conditions and preparation of cell-free extract

Twelve strains of *Lb. sanfranciscensis* were routinely propagated and cultivated in modified MRS broth containing maltose (5% w/v) and fresh yeast extract (5% v/v) at 30 °C for 24 h. Twenty-four-hours-old cells were harvested by centrifugation (13,200*g* for 10 min at 4 °C), washed twice with sterile 0.05 M potassium phosphate buffer, pH 7.0, re-suspended in sterile distilled water at a 620 nm absorbance (A_{620}) of 2.5, which corresponded to a cell concentration of ~log9.5 CFU ml^{-1}, and used for the enzyme assays.

To produce cell-free extract, washed cells were re-suspended by concentrating 25 times in 0.01 M Tris–HCl, pH 7.0 and disrupted by 10 cycles of treatment (1 min for each) with glass beads (0.1 mm in diameter) in a bead-beater (Biospec Products Inc., Hamilton Beach commercial). The glass beads were removed by decantation and disrupted cells were harvested at 16,300*g* for 15 min at 4 °C to remove cell debris. The resulting clear supernatant was dialyzed (Dialysis Tubing, cut off 12,000 Da, Sigma–Aldrich) against K-phosphate buffer, 5 mM, pH 7.0 for 24 h at 4 °C, freeze-dried and used as crude cell-free extract for purification.

2.3. Enzyme assay

PepX activity was measured in terms of *p*-nitroaniline released from glycyl-prolyl-4-nitroanilide (Gly-Pro-pNA) substrate. The assay mixture contained 900 μl of 2.0 mM Gly-Pro-pNA (final concentration) in 0.05 M

potassium phosphate buffer, pH 6.0, and 100 μl of enzyme preparation, cell-free extract or cell suspension. The mixture was incubated at 30 °C for 30 min and the absorbance was measured at 410 nm. One unit (U) of PepX activity was defined as the amount of enzyme required to liberate 1 nmol of *p*-nitroaniline per min under the assay conditions. The specific PepX activity was defined as U per mg of protein. Protein concentration in the enzyme preparations and during the purification steps was determined by the Bradford method (Bradford, 1976), using bovine serum albumin as standard. The data obtained were compared to a standard curve set up by using *p*-nitroaniline.

2.4. Enzyme purification

Six litres of a 24-h-old culture of *Lb. sanfranciscensis* CB1, cultivated in modified MRS broth, were used. After freeze-drying, the cell-free extract was re-suspended in 0.05 M potassium phosphate buffer, pH 6.0, and applied to a DEAE-cellulose anion-exchange column (55 × 1.6 cm inside diameter) (Amersham Pharmacia Biotech, Uppsala, Sweden). Proteins were eluted with a linear NaCl gradient (0–0.35 M) in 0.05 M potassium phosphate buffer, pH 7.5, at a flow rate of 55 ml h^{-1}. Fractions with the highest PepX activity were pooled, dialyzed, concentrated ~10-times by freeze-drying and subjected to gel filtration on an Ultropac TSK-gel G3000 SWXL column (600 × 7.5) (LKB-Produkter AB, Bromma, Sweden) using an automated chromatography system (consisting of a model DG-2410 degasser, SPD-6A UV detector, LC-9A pump and SIL-GA auto-injector; Shimadzu Corp., Kyoto, Japan). Elution with 0.05 M potassium phosphate buffer, pH 6.0, containing 0.15 M NaCl, was at a flow rate of 18 ml h^{-1}. Active fractions were pooled, dialyzed, concentrated ~10-times by freeze-drying and subjected to hydrophobic interaction chromatography on a FPLC Hi Trap Phenyl HP 1 ml column (Amersham Pharmacia Biotech). Proteins were eluted with a linear $(NH_4)_2SO_4$ gradient (1.0–0 M) in 0.05 M potassium phosphate buffer, pH 6.0, at a flow rate of 60 ml h^{-1}. Active fractions were pooled, dialyzed, concentrated ~10-times by freeze-drying and applied to an FPLC Mono-Q HR 5/5 column (Amersham Pharmacia Biotech) by eluting with a linear NaCl gradient from 0 to 0.5 M in the same buffer at a flow rate of 24 ml h^{-1}. Active fractions were pooled, concentrated 10 times by free-drying and, finally, subjected to gel filtration as described above.

2.5. Determination of molecular mass

The apparent molecular mass of the purified enzyme was estimated by HPLC gel filtration on the Ultropac TSKgel G3000SWXL column, using 0.05 M potassium phosphate buffer, pH 6.0, containing 0.15 M NaCl at a flow rate of 18 ml h^{-1}. Phosphorylase b (94 kDa), bovine serum albumin (67 kDa), β-lactoglobulin (18.4 kDa) and myoglobin (17.8 kDa) were used as molecular weight markers (Sigma–Aldrich). The apparent molecular mass of the purified enzyme was also estimated by SDS-PAGE according to Laemmli (1970). The gel contained 12% acrylamide (separation distance, 10 cm; gel thickness, 1 mm) and electrophoresis was performed with a Mini Protean Cell (Bio-Rad Laboratories, Dublin, Ireland). The gel was stained with silver nitrate (Sigma–Aldrich). Molecular mass marker proteins (high molecular weight standard 29.0–116.0 kDa, Sigma–Aldrich) were used as references. The SDS-PAGE gel was also cut into two parts, one of which was stained and the other was renatured in 0.05 M potassium phosphate buffer, pH 6.0, for 1 h at 30 °C, and cut into slices corresponding to the protein bands. PepX activity was detected by incubation of the slices under assay conditions.

2.6. Effects of pH and temperature on PepX activity

The optimum pH for the purified enzyme was determined at 30 °C, only in the range of 3.0–9.5, by using universal buffer. The temperature dependence was determined, at pH 6.0, only in the range 10–50 °C. To check the thermal stability of the enzyme, aliquots of the pure enzyme preparation (100 μl) were heated in capillary glass tubes at 35–45 °C for 1–30 min, and the residual activity was assayed at 30 °C and expressed as a percentage of the activity of an unheated sample. Decimal reduction time (*D*) was calculated by plotting the log of the residual activity versus time.

2.7. Substrate specificity

Substrate specificity was determined by using several *p*-nitroanilide and fluorescent substrates, and synthetic peptides. The relative activity of the PepX against *p*-nitroanilide substrates was determined according to the standard conditions described for the enzyme assay. The assay mixture used for fluorescent substrates contained 900 μl of 0.2 mM substrate (final concentration) in 0.05 M potassium phosphate buffer, pH 6.0, and 100 μl of enzyme preparation. The mixture was incubated at 30 °C and the release of fluorescence was determined after 30 min of incubation in a multiscan fluorimeter (Fluoroscan II; Labsystems, Oy, Finland) at excitation and emission wavelengths of 360 and 440 nm, respectively. The assay conditions used for peptides was the same as that for *p*-nitroanilide substrates. The assay mixture used to detect the hydrolysis of 33-mer peptide contained 133 μl of 0.2 mM substrate (final concentration) in 0.05 M potassium phosphate buffer, pH 6.0, and 66.7 μl of enzyme preparation. The mixture was incubated at 30 °C for 24 h. Leucine aminopeptidase (Sigma–Aldrich) was also used (1.5 U/ml, final concentration) together

with the PepX preparation to determine the synergy of activity toward the 33-mer peptide. The individual effect of the leucine aminopeptidase toward the 33-mer peptide was determined by incubation without PepX preparation under the same conditions.

The hydrolysis of peptide substrates was determined with a BioChrom 30 series Amino Acid Analyser (Biochrom LTD, Cambridge Science Park, England) using a Na cation exchange column (20×0.46 cm, i.d.) and amino acids were post-column derivatized with ninhydrin reagent and detected by absorbance at 440 (proline and hydroxyproline) or 570 nm (all the other amino acids) (Di Cagno et al., 2004).

The hydrolysis of the 33-mer peptide was determined with the Amino Acid Analyser and by reverse-phase fast performance liquid chromatography (RP-FPLC) using a Resource RPC 3 ml column and FPLC equipment with a UV detector operating at 214 nm (Amersham Pharmacia Biotech). Elution was at a flow rate of 1.0 ml min^{-1} with a gradient (5–100%) of acetonitrile (CH_3CN) in 0.05% trifluoroacetic acid. The concentration of CH_3CN was increased from 5% to 46% between 16 and 62 min and from 46% to 100% between 62 and 72 min.

2.8. Determination of K_m and V_m

K_m value for the activity of PepX on Gly-Pro-pNA was estimated at 30 °C, using a range of concentration from 0.025 to 2.0 mM. A Lineweaver–Burk plot (1934) was used for the determination.

2.9. Effect of inhibitors and divalent cations on enzyme activity

To assay the effects of inhibitors and divalent cations, a mixture containing the partially purified enzyme and 10 mM (final concentration) chemical reagents or divalent cations in 0.05 M potassium phosphate buffer, pH 6.0, was incubated for 30 min at 30 °C. Reaction was initiated by adding the appropriate substrate, and enzyme activity was assayed under standard conditions. Controls to eliminate the interference of inhibitors or divalent cations were included.

2.10. Determination of inhibition kinetic analysis

The analysis of the inhibition constants for PepX was done in the presence of the most effective inhibitors. The mixture reactions contained Gly-Pro-pNA in a concentration range of 0.025–2 mM without inhibitor or in the presence of inhibitors at three different concentrations, namely 1,10-phenanthroline (1, 10 and 20 mM), 3,4-dichloroisocoumarin (1, 10 and 20 mM), and PCMB (1, 5 and 10 mM). The inhibition mode of action was determined from a Lineweaver–Burk plot, $1/V_0$ activity against 1/[S]. The K_i value was determined from the slope of the Lineweaver–Burk plot against the concentration of corresponding inhibitor, as decribed by Segel (1976).

2.11. Experimental design and statistical analysis

The effects of temperature, pH and NaCl on the PepX activity on Gly-Pro-pNA were determined by modulating the variables according to a three factors, five levels Central Composite Design (CCD). The 17 combinations obtained for each enzyme activity are shown in Table 1. Two replicates of each combination were used.

Modelling was aimed at describing the enzyme activity as a function of the independent variables of the CCD. A software package (Statistica for Windows, Statsoft, Tulas, OK) was used to fit the second order model to the independent variables using the following equation:

$$\gamma = \sum B_i \chi_i + \sum B_{ii} \chi_i^2 + \sum B_{ij} \chi_i \chi_j,$$

where γ is the dependent variable to be modelled, B_i, B_{ii} and B_{ij} are regression coefficients of the model, and χ_i and χ_j are the independent variables in coded values. The variables with significance below 95% ($P > 0.05$) were not included in the final model. The experimental data were modelled using stepwise regression with all variables in the model and the non-significant terms were omitted one-by-one, consequently only terms significant at 95% were considered. The three dimensional surface plot was drawn to illustrate the main and interactive effects of the independent variables (temperature, pH and NaCl) on the dependent one (enzyme activity).

Table 1
Compositions of the various runs of the Central Composite Design and specific activity (U[a]/mg of protein) of the X-prolyl dipeptidyl aminopeptidase (PepX) of *Lactobacillus sanfranciscensis* CB1

	Temperature (°C)	pH	NaCl (%)	U/mg
1	26	4.1	1.2	5.70
2	26	5.5	1.2	25.2
3	26	4.1	3.6	4.10
4	26	5.5	3.6	23.8
5	38	4.1	1.2	4.10
6	38	5.5	1.2	11.9
7	38	4.1	3.6	3.30
8	38	5.5	3.6	11.2
9	32	4.8	2.4	11.9
10	32	4.8	2.4	11.9
11	32	3.4	2.4	3.30
12	32	6.2	2.4	33.2
13	32	4.8	0	25.7
14	32	4.8	4.8	9.50
15	20	4.8	2.4	17.8
16	44	4.8	2.4	3.50
17	32	4.8	2.4	12.3

The specific activity was the average of three replicates of each combination.

[a] U, is the amount of enzyme required to liberate 1 nmol of *p*-nitroaniline per min under the assay conditions.

3. Results and discussion

3.1. PepX activity of Lb. sanfranciscensis strains

Recently, Di Cagno et al. (2004, 2002) selected four sourdough lactobacilli (*Lb. sanfranciscensis*, *Lactobacillus brevis*, *Lactobacillus alimentarius* and *Lactobacillus hilgardii*) which showed a considerable hydrolysis of albumin, globulin and gliadin fractions during wheat sourdough fermentation. The pool of these lactobacilli had the capacity to hydrolyze various Pro-rich oligopeptides, including 33-mer peptide, fragment 62–75 of the α2-gliadin and fragment 31–43 of the A-gliadin. Acute in vivo challenges, based on intestinal permeability, showed the tolerance of coeliac patients to breads fermented by selected lactobacilli which contained 2 g of gluten (Di Cagno et al., 2004). Nevertheless, the enzyme activities of these selected sourdough lactobacilli were not characterized. Simpson (2001) proposed the wheat flour endogenous PepX as a crucial enzyme for the degradation of Pro-rich oligopeptides and other authors (Hausch et al., 2002) have supposed that gastrointestinal PepX may have a role in the degradation of Pro-rich oligopeptides responsible for coeliac disease.

Preliminarily, the cell suspensions (~10^9 cfu/ml) of 12 strains of *Lb. sanfranciscensis*, isolated from sourdoughs (Corsetti et al., 2001; Gobbetti, Corsetti, Rossi, La Rosa, & De Vincenzi, 1994), were screened for PepX activities using Gly-Pro-pNA as substrate (Table 2). Although, with some differences, the activity was widely distributed across all the *Lb. sanfranciscensis* strains, after 30 min of incubation at 30 °C, the highest activities were found for *Lb. sanfranciscensis* 22Z, 9M, 22E, 13R, 14C and CB1, which ranged from 63.3 to 57.0 U/ml. The other strains showed activities of less than 55.4 U/ml. Based on these preliminary results and by considering that *Lb. sanfranciscensis* CB1 is a well known strain, partially characterized for its proteolytic system (Gobbetti et al., 1996a; Gobbetti, Smacchi, Fox, Stepaniak, & Corsetti, 1996b) and for other metabolic properties (De Angelis et al., 2003, 2002; Gobbetti, 1998), it was used for the further characterization of the PepX activity.

Table 2
X-prolyl dipeptidyl aminopeptidase (PepX) activity (U^A) of *Lactobacillus sanfranciscensis* strains after 30 min of incubation at 30 °C in modified MRS broth

Strains	PepX activity (U/ml)
Lb. sanfranciscensis 14C	$58.5^{c,d}$
Lb. sanfranciscensis 9M	$61.7^{a,b}$
Lb. sanfranciscensis 22E	$60.1^{b,c}$
Lb. sanfranciscensis CB1	$57.0^{d,e}$
Lb. sanfranciscensis 9F	53.5^{f}
Lb. sanfranciscensis 13R	$60.1^{b,c}$
Lb. sanfranciscensis 13A	39.3^{h}
Lb. sanfranciscensis 22Z	63.3^{a}
Lb. sanfranciscensis 5D	54.1^{f}
Lb. sanfranciscensis 7H	9.15^{i}
Lb. sanfranciscensis 7A	42.2^{g}
Lb. sanfranciscensis 20C	$55.4^{e,f}$

The X-prolyl dipeptidyl aminopeptidase activity was the average of three replicates twice analyzed.

[a–h] Values in the same column with different superscript letters differed significantly ($P < 0.05$).

[A] U, is the amount of enzyme required to liberate 1 nmol of *p*-nitroaniline per min under the assay conditions.

3.2. Partial purification and molecular mass determination

The PepX of *Lb. sanfranciscensis* CB1 was partially purified using five chromatographic steps: anion-exchange on DEAE-cellulose, gel filtration on HPLC TSKgel G3000SWXL, hydrophobic interaction on FPLC Hi Trap Phenyl HP 1 ml, anion-exchange on Mono-Q HR 5/5 and again gel filtration on HPLC TSK-gel G3000SWXL columns. The cell-free extract and the pooled active fractions, after each chromatographic step, were analyzed by SDS-PAGE (Fig. 1(a)). After the final chromatographic step on gel filtration, four protein bands were detected by SDS-PAGE (Fig. 1(a)) with apparent molecular masses which ranged from 49.2 to 66.0 kDa. On excision from the SDS-PAGE

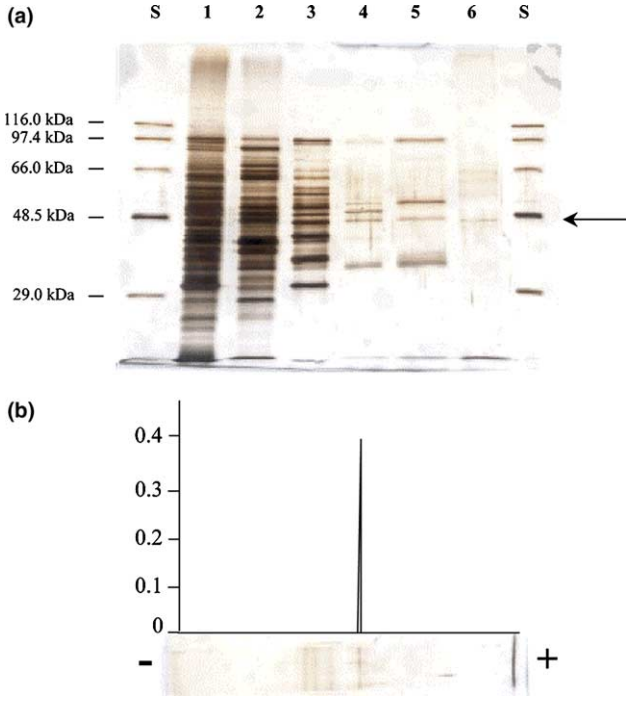

Fig. 1. (a) SDS-PAGE for the intracellular X-prolyl-dipeptidyl aminopeptidase (PepX) of *Lactobacillus sanfranciscensis* CB1. Lanes: S, reference proteins; 1, cytoplasmic extract; 2, enzyme preparation after anion-exchange on DEAE-cellulose; 3, after gel filtration on TSK G3000 SWXL; 4, after hydrophobic interaction on Hi Trap Phenyl HP; 5, after anion-exchange on Mono-Q HR 5/5; and 6, after gel filtration on TSK G3000 SWXL. (b) Enzyme activity of the corresponding region of gel excised before staining.

gel, only the protein band with the lowest apparent molecular mass had PepX activity (Fig. 1(b)).

The apparent molecular mass for the PepX of *Lb. sanfranciscensis* CB1 was estimated to be ~49.2 kDa by SDS-PAGE (Fig. 1(a)) and ~56.0 kDa by gel filtration (data not shown), indicating that the enzyme exists as a monomeric protein. This finding differed from those reported for dairy lactic acid bacteria. PepX enzymes of *Lactococcus lactis* subsp. *cremoris* (Kiefer-Partsch, Bockelmann, Geis, & Teuber, 1989), *Lc. lactis* subsp. *lactis* (Lloyd & Pritchard, 1991; Zevaco, Monnet, & Gripon, 1990), *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. acidophilus* (Bockelmann et al., 1991) were found to be dimers of 150–190 kDa. Nevertheless, monomeric configurations of 72–87 kDa was reported for *Lb. helveticus* (Miyakawa et al., 1994) and *Lb. casei* subsp. *casei* (Habibi-Najafi & Lee, 1994).

3.3. Effects of pH and temperature

The activity of the PepX was optimal at pH 6.0. It maintained 50–80% of the maximum activity in the range of pH 5.0–5.5 and 60–70% at pH 6.5–7.5. Overall, the only endogenous PepX isolated from cereals (barley) had a higher optimum pH (~7.2) (Simpson, 2001) as also did the corresponding PepX of dairy lactic acid bacteria (pH 6.5–7.5) (Bockelmann et al., 1991; Habibi-Najafi & Lee, 1994; Khalid & Marth, 1990; Kiefer-Partsch et al., 1989; Meyer & Jordi, 1987; Miyakawa et al., 1994, 1991; Sanz & Toldrá, 2001; Tsakalidou, Anastasiou, Papadimitriou, Manolopoulou, & Kalantzopoulos, 1998; Vesanto et al., 1995). The optimum temperature of the PepX of *Lb. sanfranciscensis* CB1 was found to be 30 °C, with ~70% of residual activity at 25 and 35 °C. This value is lower than the range of 40–55 °C which characterized the optimal activities of PepX from dairy lactic acid bacteria (Bockelmann et al., 1991; Degraeve & Martial-Gros, 2003; Habibi-Najafi & Lee, 1994; Khalid & Marth, 1990; Kiefer-Partsch et al., 1989; Meyer & Jordi, 1987; Miyakawa et al., 1994, 1991; Sanz & Toldrá, 2001; Tsakalidou et al., 1998; Vesanto et al., 1995; Zevaco et al., 1990). Based on the above findings, it seemed that PepX of *Lb. sanfranciscensis* is well adapted to temperature and acid conditions that characterize the sourdough fermentation. The thermal stability of PepX of *Lb. sanfranciscensis* CB1 was determined at 35, 40 and 45 °C. It showed a rapid inactivation after treatment for 20 min at 45 °C. The *D* value calculated at 45 °C was ~5.46 s. A rapid enzyme inactivation above 50 °C has been described for several PepX of dairy lactic acid bacteria (Bockelmann et al., 1991; Habibi-Najafi & Lee, 1994; Miyakawa et al., 1991).

3.4. Substrate specificity

As shown in Table 3, the specificity of the partially purified PepX of *Lb. sanfranciscensis* CB1 was essentially confined to substrates which contained proline at the N-penultimate position (Gly-Pro-pNA and Gly-Pro-AMC substrates). The same activity was confirmed toward the tripeptide, Gly-Pro-Ala (58.7 μmol min^{-1}). Highest activity toward peptide bonds with X-Pro as N-terminal sequence was also found for the PepX of *Streptococcus thermophilus* (Tsakalidou et al., 1998). The PepX of *Lb. sanfranciscensis* CB1 did not show prolidase (Gly-Pro) or aminopeptidase (Pro-pNA and Pro-AMC) activities. The lack of hydrolysis of the substrate Suc-Gly-Pro-Leu-Gly-Pro-AMC also indicated that the partially purified enzyme did not possess endopeptidase activity. This result was confirmed by using the epitope the 33-mer peptide (L-Q-L-Q-P-F-P-Q-P-Q-L-P-Y-P-Q-P-Q-L-P-Y-P-Q-P-Q-L-P-Y-P-Q-P-Q-P-F) (0.2 mM); after 24 h of incubation no hydrolysis was found by PepX.

Byun et al. (2001) described the synergistic effect of PepX and non-specific aminopeptidase (type N), and their activity in boosting the degree of hydrolysis of gluten. Therefore, the synergistic activity of PepX of *Lb. sanfranciscensis* CB1 and leucine aminopeptidase (Sigma–Aldrich) was assayed. Under these conditions, the hydrolysis of the 33-mer peptide was complete in 24 h (0.138 μmol min^{-1}). As shown by the analysis of free amino acids, the incubation (24 h) of the epitope 33-mer with leucine aminopeptidase alone liberated leucine and glutamine residues only, and no traces of proline or other amino acids were found. Leucine and glutamine are the NH_2-terminal residues of 33-mer peptide. Hausch et al. (2002) reconstructed the digestive break-

Table 3
Substrate specificity (%) of the X-prolyl-dipeptidyl aminopeptidase (PepX) of *Lactobacillus sanfranciscensis* CB1 on different substrates

Substrate	Relative activity (%)[A]	Substrate	Relative activity (%)[A]
Gly-Pro-pNA	100[a]	Gly-Pro-AMC	100[a]
L-Glu-pNA	0.34[b]	Pro-Arg-AMC	1.38[b]
Leu-pNA	0.00[b]	Suc-Gly-Pro-Leu-Gly-Pro-AMC	0.37[c]
Pro-pNA	0.00[b]	Pro-AMC	0.00[c]

The X-prolyl dipeptidyl aminopeptidase activity was the average of three replicates twice analyzed.

[a–c] Values of *p*-nitroanilide or fluorescent substrates in the same column with different superscript letters differed significantly ($P < 0.05$).

[A] The rates of hydrolysis of Gly-Pro-pNA and Gly-Pro-AMC were taken as 100% and the relative activities of *p*-nitroanilide and fluorescent substrates were, respectively, referred to them.

down of the peptide 57–68 (QLQPFPQPQLPY) of the α9-gliadin which has been shown to stimulate the proliferation of T cells isolated from coeliac patients (Arentz-Hansen et al., 2000). In agreement with our findings, the preferred pathway involves serial cleavages of the NH_2-terminal glutamine and leucine residues by aminopeptidase N, followed by hydrolysis of the remaining NH_2-terminal Q-P-dipetide by PepX. The same authors (Hausch et al., 2002) showed that the three major intermediate products (QPFPQPQLPY, QPFPQPQLP, FPQPQLP) released during digestion of QLQPFPQPQLPY are substrates for PepX. As previously reported (Gobbetti et al., 1996a), *Lb. sanfranciscensis* CB1 also possesses a non-specific aminopetidase type N that, together with PepX, could permit the efficient hydrolysis of the 33-mer peptide or of other Pro-rich oligopeptides during sourdough fermentation. The epitope 33-mer is probably the most important inducer of gut-derived human T-cell lines in coeliac patients for several reasons: (i) it remains intact despite prolonged exposure to gastric and pancreatic proteases; (ii) other patient-specific T-cell epitopes are present in its sequence; (iii) hydrolysis of the 33-mer peptide (100 μM) by small intestinal brush-border membrane enzymes is less than 20% over 20 h of incubation, and (iv) it remains intact for a long time (~24 h) in the small intestine and even at low concentration is able to act as a potential antigen for T-cell proliferation and intestinal toxicity in genetically susceptible individuals (Shan et al., 2002). The hydrolysis of the 33-mer peptide by lactobacilli during sourdough fermentation may be a novel tool to manage coeliac disease. The synergistic activity of PepX and aminopeptidase type N may partially explain the results on the tolerance of coeliac patients to breads, fermented by selected lactobacilli, which contained 2 g of gluten (Di Cagno et al., 2004).

3.5. K_m and V_m determinations

As determined by the linear Lineweaver–Burk plot of $1/V$ against 1/[S] on Gly-Pro-pNA, the values of K_m and V_{max} for the PepX of *Lb. sanfranciscensis* CB1 were 0.6 mM and 99.6 nmol mg^{-1} min^{-1}, respectively. These values differed from those determined for *Lb. helveticus* (K_m and V_{max} of 0.24 mM and 5.0 nmol mg^{-1} min^{-1}, respectively) (Degraeve & Martial-Gros, 2003), *Lb. sakei* (K_m and V_{max} of 0.029 mM and of 47.44 μmol mg^{-1} min^{-1}, respectively) (Sanz & Toldrá, 2001) and *Str. thermophilus* (K_m and V_{max} of 3.1 mM and 3500 μmol mg^{-1} min^{-1}, respectively) (Tsakalidou et al., 1998) on the same substrate.

3.6. Effect of inhibitors and divalent cations

Table 4 shows the effects of inhibitors and divalent cations on the PepX activity of *Lb. sanfranciscensis* CB1. 3,4-Dichloroisocoumarin, which reacts with serine groups, caused a decrease of ~90% of the maximum activity. Nevertheless, PMSF, which has the same specificity as 3,4-dichloroisocoumarin, had no effect. Under the same conditions, 1,10-phenanthroline caused a decrease of ~90%, while EDTA showed a lower effect of inactivation (~40%). Among chemicals which react with sulphydryl groups, *N*-ethylmaleimide and iodoacetamide had no effect. Incubation with *p*-chloromercuric benzoic acid yielded a considerable inactivation of the PepX activity. This indicates the possible involvement of functional sulfhydryl group(s) at or near the active site of the enzyme. Although all the PepX of dairy lactic acid bacteria cited in the literature are considered to be serine enzymes, many authors have claimed that sulphydryl groups may also be important for the enzyme activity (Miyakawa et al., 1994, 1991). The above spectrum of inhibition was rather similar to that found for the PepX of *Str. thermophilus* (Tsakalidou et al., 1998). Among divalent cations, only Zn^{2+}, Hg^{2+} and Mn^{2+} markedly decreased the activity of the PepX of *Lb. sanfranciscensis* CB1.

Table 4
Effects of inhibitors and divalent cations on the X-prolyl-dipeptidyl aminopeptidase (PepX) activity of *Lactobacillus sanfranciscensis* CB1

Inhibitors and cations[a]	Relative activity[b] (%)
3,4-Dichloroisocoumarin	10.7 ± 0.4
PMSF	101 ± 0.6
1,10-Phenanthroline	9.43 ± 0.7
EDTA	66.5 ± 1.9
NEM	124 ± 0.2
Iodacetamide	139 ± 0.2
PCMB	0.00 ± 0.1
DTT	124 ± 3.0
Mg^{2+}	87.3 ± 2.0
Cu^{2+}	42.0 ± 3.2
Co^{2+}	56.0 ± 3.0
Hg^{2+}	10.0 ± 2.6
Mn^{2+}	29.8 ± 3.5
Ca^{2+}	117 ± 2.2
Fe^{2+}	40.6 ± 3.3
Zn^{2+}	4.94 ± 0.7

The X-prolyl dipeptidyl aminopeptidase activity was the average of three replicates twice analyzed.

[a] PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetra acetic acid; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate; DTT, dithiothreitol.

[b] The activity of the control in the absence of chemical reagents and divalent cations was taken as 100%.

3.7. Inhibition kinetic analysis

According to the procedure of Lineweaver & Burk (1934), the kinetics of inhibition were determined for the strongest inhibitors: *p*-chloromercuric benzoic acid, 3,4-dichloroisocoumarin and 1,10-phenanthroline (Table 5). The three compounds were found to be competitive inhibitors. PCMB was the most potent

Table 5
Inhibition modes and inhibition constants (K_i) of selected inhibitors for the X-prolyl-dipeptidyl aminopeptidase (PepX) activity of *Lactobacillus sanfranciscensis* CB1

Inhibitors	Inhibitor concentration (mM)	K_i (mM)[a]	Inhibition mode[b]
3,4-Dichloroisocoumarin	1	0.45	Competitive
	10	0.26	
	20	0.35	
1,10-Phenanthroline	1	0.30	Competitive
	10	0.12	
	20	0.04	
PCMB	1	0.13	Competitive
	5	0.06	
	10	0.06	

[a] Determined from the slope of the reciprocal plot against the inhibitor concentration.
[b] Determined from the Lineweaver–Burk plot of $1/V$ against 1/[Gly-Pro-pNA].

inhibitor with a K_i of 0.06 mM, followed by 1,10 phenanthroline and 3,4-dichloroisocoumarin. This mode of inhibition explains that *p*-chloromercuric benzoic acid, 3,4-dichloroisocoumarin and 1,10-phenanthroline bind only to the active site of the free enzyme, prohibiting the substrates from undergoing hydrolysis.

3.8. Experimental design and statistical analysis

The CCD was used because it reduces the number of possible combinations to a manageable size, using only a fraction of the total number of factor combinations for experimentation. In statistical literature, this technique is known as confounding (Gacula, 1988). Ranges of temperature (20–44 °C), pH (3.4–6.2) and NaCl (0–4.8%), which are compatible with the sourdough processing (Gobbetti, 1998), were considered. The polynomial equation describing the effects of the different variables on the PepX activity of *Lb. sanfranciscensis* CB1 was the following: $-1.778[\mathrm{NaCl}] - 0.123[\mathrm{pH}][T] + 1.53[\mathrm{pH}]^2$, with a regression coefficient, R, of 0.975, an F-value of 89.266 and a standard error of residuals, SE of 3.848.

The effects indicated by the equation are better shown by considering the three-dimensional plots of Fig. 2. They were obtained by imposing a constant value (e.g., the central point of the CCD) on one variable at a time. The highest activity (~40 U/mg) was found at 20 °C, pH 6.2 and 2.4% NaCl (Fig. 2(a)). At the highest value of pH (6.2) the increase of temperature up to 44 °C caused only a limited decrease of the PepX activity (Fig. 2(a)), while the lowest values of pH (below 4.8) always had a marked negative effect (Fig. 2(a) and (b)). Also, the increase of the concentration of NaCl from 0% to 4.8% only slightly affected the PepX activity when the value of pH was near to the optimum (pH 6.0) (Fig. 2b). When temperature and NaCl interacted at pH 4.8 (Fig. 2(c)), they did not cause noticeable variations of the PepX activity. At 32 °C, pH 5.5 and 2.4% NaCl, the activity was still maintained at ~20 U/mg, confirming the efficiency of the PepX of *Lb. sanfranciscensis* CB1 during sourdough fermentation.

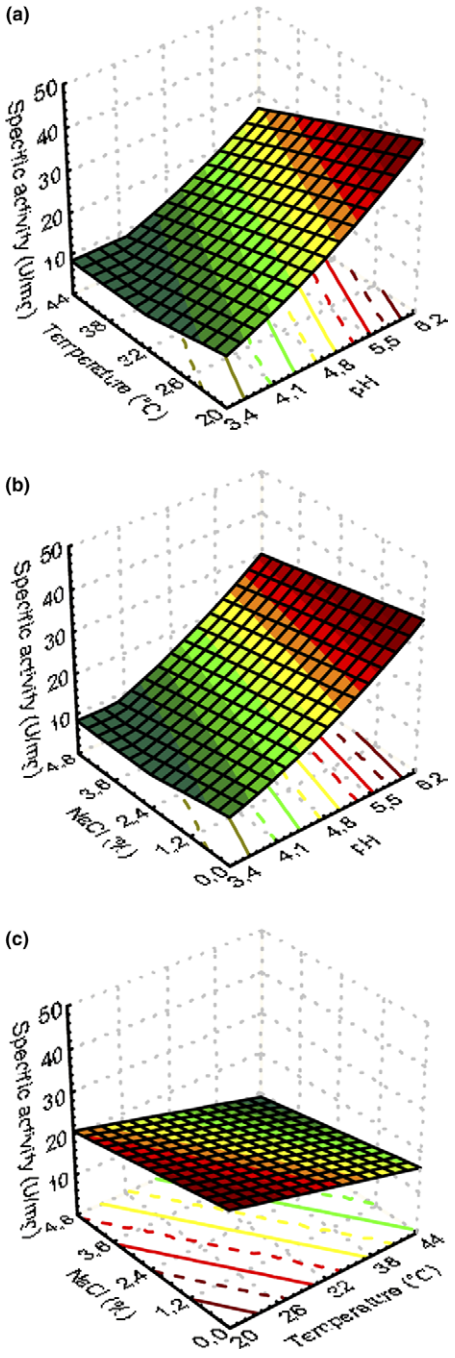

Fig. 2. X-prolyl-dipeptidyl aminopeptidase (PepX) activity of *Lactobacillus sanfranciscensis* CB1. Three-dimensional plots of the interactions of: (a) temperature × pH; (b) pH × NaCl; (c) temperature × NaCl.

Lb. sanfranciscensis CB1 has been shown to have a particular capacity to degrade proteins or peptides during sourdough fermentation and serine proteinase, me-

tal-dependent dipeptidase, and general aminopeptidase have been purified and characterized previously (Gobbetti et al., 1996a, 1996b). This study provides substantial information on the biochemical characteristics and potential role of the PepX of *Lb. sanfranciscensis* CB1. Besides its potential role in microbial physiology and in flavour development during sourdough fermentation and baking, the PepX of *Lb. sanfranciscensis* CB1, in a synergistic way with flour and/or microbial general aminopeptidase, may be the crucial enzymes to hydrolyze Pro-rich oligopeptides, which are responsible for coeliac disease.

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