Characterization of the Recombinant Exopeptidases PepX and PepN from *Lactobacillus helveticus* ATCC 12046 Important for Food Protein Hydrolysis

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Abstract

The proline-specific X-prolyl dipeptidyl aminopeptidase (PepX; EC 3.4.14.11) and the general aminopeptidase N (PepN; EC 3.4.11.2) from *Lactobacillus helveticus* ATCC 12046 were produced recombinantly in *E. coli* BL21(DE3) via bioreactor cultivation. The maximum enzymatic activity obtained for PepX was 800 μkatH-Ala-Pro-pNA L⁻¹, which is approx. 195-fold higher than values published previously. To the best of our knowledge, PepN was expressed in *E. coli* at high levels for the first time. The PepN activity reached 1,000 μkatH-Ala-pNA L⁻¹. After an automated chromatographic purification, both peptidases were biochemically and kinetically characterized in detail. Substrate inhibition of PepX and product inhibition of both PepX and PepN were discovered for the first time. An apo-enzyme of the Zn²⁺-dependent PepN was generated, which could be reactivated by several metal ions in the order of Ca²⁺>Zn²⁺>Mn²⁺>Ca²⁺>Mg²⁺. PepX and PepN exhibited a clear synergistic effect in casein hydrolysis studies. Here, the relative degree of hydrolysis (rDH) was increased by approx. 132%. Due to the remarkable temperature stability at 50°C and the complementary substrate specificities of both peptidases, a future application in food protein hydrolysis might be possible.

Introduction

Enzymatic protein hydrolysis is a complex process. The synergistic effects of various peptidases are necessary to achieve a reasonable degree of hydrolysis. Enzymatic hydrolysis of food proteins is widely used to increase the value of the final products by improving its nutritional characteristics, adding functional properties and removing inhibitory peptides [1,2]. Furthermore, hydrolyzed protein formulas reduce or eliminate allergenicity and problem that occurs during food protein hydrolyses [13,14]. Neither have any inhibition studies utilizing PepX from different *Lb. helveticus* strains [5,11,12] or other LAB exopeptidases been reported. Product inhibition for endopeptidases is a known problem that occurs during food protein hydrolyses [13,14].

We report the production of recombinant PepN and PepX from *Lb. helveticus*. Both recombinant enzymes were purified and biochemically characterized. Kinetic studies were also performed to investigate substrate and product inhibition. The synergistic effects of PepN and PepX in casein hydrolysis were investigated.

Materials and Methods

Materials

All chemicals (analytical grade) were obtained from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany), Carl Roth (Karlsruhe, Germany), AppliChem GmbH (Darmstadt, Germany), or Merck AG (Darmstadt, Germany). Oligonucleotides were purchased from biomers.net GmbH (Ulm, Germany). Molecular biology kits and enzymes were obtained from Qiagen (Hilden, Germany), Fermentas GmbH (St. Leon-Rot, Germany), New England Biolabs GmbH (NEB, Frankfurt am Main, Germany), or Roche (Mannheim, Germany). Chromogenic peptides and other standard peptides were purchased from...
Bacterial Strains and Culture Conditions

The strain Lactobacillus helveticus ATCC 12046 was cultivated in de Man, Rogosa and Sharpe (MRS) medium [15] with constant shaking at 37 °C. E. coli DH5α (Invitrogen, Carlsbad, USA) and E. coli BL21(DE3) (Novagen, Madison, USA) strains were used as hosts for plasmid maintenance and T7 expression work, respectively. Standard protocols were used for the preparation and transformation of competent E. coli cells with plasmid DNA via heat shock [16]. Cells were cultivated in Luria Bertani (LB) medium supplemented with the appropriate antibiotic (100 μg mL⁻¹ ampicillin) and agar (15 g L⁻¹) for agar plates. All cultures were grown with continuous shaking at 37 °C unless otherwise stated.

Cloning, Construction of Expression Vectors and Sequencing of pepX and pepN

The total genomic DNA from Lb. helveticus ATCC 12046 was extracted using an identical method as previously described [17]. Polymerase chain reaction (PCR) was performed using HotStar HiFidelity polymerase (Qiagen), according to the manufacturer’s instructions. The primers pepX_for (5'-GGGGATCCATGAAATGGAATAAACACCAATATG-3') and pepX_rev (5'-CGGATGTAGGATTTATATTTTATATAA-3') were used for the amplification of the pepX gene based on the nucleotide sequence of pepX from Lactobacillus helveticus CNRZ 32 (EMBL: AAB50275) that is available in the UniProt database (UniProt ID: Q59485). The PCR product (approx. 2,400 bp) of pepX (2,379 bp) was cloned into the pJET1.2 vector (Fermentas), according to the manufacturer’s instructions. Similarly, the pepN gene was amplified with the primers Ndel_pepN_for (5'-AGAGCTTCGATATGAAATTACCAATATG-3') and XhoI_pepN_rev (5'-ACTAATCTCGAGTTTTTCATAAAACTT-3') for the cloning of the pepN gene based on the nucleotide sequence of pepN from Lactobacillus helveticus (EMBL: CTK51574) that is available in the UniProt database (UniProt ID: Q10739), resulting in an approx. 2,500 bp PCR product.

The PCR products of pepX and pepN (genomic DNA as template) were purified (QiAquick Gel Extraction Kit; Qiagen) and sequenced by Sanger sequencing (700 bp). The nucleotide sequence analyses and database searches were conducted, as described previously [17].

Expression of Recombinant PepX and PepN in E. coli BL21(DE3)

Transformed E. coli BL21(DE3) strains were grown in 2× YT medium that contained glucose (10 g L⁻¹) supplemented with ampicillin (100 μg mL⁻¹). Precultures were incubated at 37 °C on a rotary shaker. The first precultures were cultivated for 18 h and the second precultures for 13 h. The main cultures (800 mL) were grown in a bioreactor parallel system (Multifors), following the analytical methods previously described [17], with some modifications. The stirrer speed varied between 500 and 1000 rpm. The temperature was maintained at 30 °C until the OD₆₀₀ reached a value of 5 to minimize the formation of inclusion bodies, and protein expression was induced by the addition of 0.5 mM IPTG. During the cultivations, samples were removed at various time points, and the enzymatic activity was determined from the cell-free extract after cell disruption [17]. The cultures were harvested after 23 h of cultivation, as previously described [17].

Automated Purification of PepX and PepN

Both PepX and PepN were individually purified using Ni²⁺ immobilized metal affinity chromatography (IMAC) and subsequently desalted via two HiPrep™ 26/10 columns using an automated operating procedure, as previously reported [17,18]. Cell suspensions of 15% (v/v) were prepared in 50 mM Na₂HPO₄/KH₂PO₄ buffer (pH 6.3) containing 500 mM NaCl and 20 mM imidazole (PepX) or 10 mM imidazole (PepN). Both enzymes were eluted by increasing the imidazole concentration to 500 mM in an identical buffer. Subsequently, the enzymes were desalted in 30 mM Na₂HPO₄/KH₂PO₄ buffer (pH 6.3).

Polyacrylamide Gel Electrophoresis (PAGE)

The samples, following cell disruption (sonication), were divided into soluble and insoluble fractions. These samples and purified PepX and PepN (5 μg of protein each; [19]) were analyzed by sodium dodecyl sulfate (SDS) PAGE (12.5% gel) [20]. A standard molecular weight protein mixture was used as a reference (NEB). Gels were stained with Coomassie Brilliant Blue to detect the proteins.

Native PAGE (8% gel) was conducted on ice (4 °C) with soluble samples following purification (5 μg of protein each; [19]). A native standard molecular weight protein mixture was obtained from SERVA Electrophoresis GmbH (Heidelberg, Germany) and was used as a reference. Gels were stained with Coomassie Brilliant Blue to detect the proteins. A solution was prepared for activity staining containing H-Ala-Pro-pNA (PepX) or H-Ala-pNA (PepN) (5 mg of each) dissolved in dimethylformamide (DMF, 250 μL) and diluted to a final volume of 10 mL with Na₂HPO₄/KH₂PO₄ buffer (50 mM; pH 6.5). The lanes of the native PAGE gel used for activity staining were excised and then incubated with the corresponding pNA solution at 37 °C until yellow bands were observed. The yellow bands developed were redyed to violet-colored bands using the following protocol [21]. The lanes of the native PAGE gel containing yellow bands were incubated in a 0.1% (w/v) NaNO₂/1 M HCl solution until the bands disappeared (approx. 2 min). The lanes were then washed with 1% (w/v) urea/HCl for 30 sec. Finally, the bands were developed by incubating the native PAGE lanes in 0.025% (w/v) N,N-diethyl-ethylene diamine dihydrochloride/22% (v/v) EtOH for approx. 10 min, allowing the violet color to appear. The native PAGE gel was digitally imaged before the violet color disappeared (approx. 5–30 min).

Measurement of Proteolytic Enzymatic Activity Using Chromogenic Peptides

The chromogenic peptides H-Ala-Pro-pNA and H-Ala-pNA were used to determine the proteolytic activities of PepX and PepN, respectively, in a standard assay. The standard assay was performed as follows. Initially, 50.5 μL of the enzyme solution was added to 199.5 μL of the buffer 1×. After incubation (15 min) at 37 °C, the reaction was stopped by adding 100 μL of the buffer 1×. The reaction was initiated by the addition of 10 μL of the substrate 1× (H-Ala-Pro-pNA or H-Ala-pNA). The reaction was monitored for 10 min at 405 nm. The UV-absorbance was measured in a UV/VIS spectrophotometer (Ultrospec 3300 pro, Amersham Biosciences). The proteolytic activity was calculated as the amount of substrate degraded per minute and per milligram of protein. The proteolytic activities of PepX and PepN were expressed as umol min⁻¹ mg⁻¹ of protein.
Characterization of the Exopeptidases PepX & PepN

The purified PepX and PepN (see above) were characterized. All experiments were repeated at least once, and the data reported were measured in triplicate. The error bars were not included in the graphical representations if the standard deviation was below 5%.

**Determination of enzyme kinetics.** The kinetic parameters were determined using H-Ala-Pro-pNA, H-Arg-Pro-pNA and H-Gly-Pro-pNA · p-tosylate as the substrates for PepX. The substrates H-Arg-pNA · 2HCl, H-Lys-pNA · 2HBr, H-Leu-pNA, H-Ala-pNA, H-Phe-pNA, H-Val-pNA, H-Ile-pNA, H-Gly-pNA, H-Pro-pNA, H-His-pNA, and H-Glu-pNA were used for PepN. Standard enzymatic activity assay conditions were used in which the substrate concentrations ranged from 0.017 to 7.47 mM, depending on the particular enzyme and substrate. The enzyme solution was preincubated with the inhibitor at 37 °C for 15 min for product inhibition kinetic studies.

**Effect of pH and temperature on the enzymatic activity.** The activity was assayed under standard conditions in which the enzymes were diluted and the assay buffer was replaced by the corresponding buffer to determine the pH profiles of PepX and PepN. The pH values ranged from 5 to 10.

The activities were assayed under standard conditions in which the assay temperatures ranged from 10 to 65 °C (PepX) or from 10 to 55 °C (PepN) to assess the effect of temperature on the enzymatic activity. The enzyme solutions were incubated at different temperatures (0 to 50 °C) for 15 d to determine the thermal stability. Subsequently, the residual activity was measured under standard conditions (see above).

**Effect of inhibitors, cations, metal chelators, reducing agents, and solvents on the enzymatic activity.** The substances tested were solubilized and diluted in H2O4Odimethyl sulfoxide (DMSO), acetone, or ethanol, depending on the substance. The assay conditions were identical to the standard protocol, except that 24 μL of the chromogenic peptide solution (5 mg mL−1 for PepX; 7.5 mg mL−1 for PepN, dissolved in DMSO) was added to the reaction mixture. The reaction was terminated by adding 50 μL of 50% (v/v) acetic acid. After centrifugation (8,000 × g, 5 min), 240 μL of the solution was transferred to a microtiter plate, and the absorption was measured in an iEMS Reader (Labsystems, Helsinki, Finland) at 405 nm. One katal of proteolytic activity was defined as the amount of enzyme required to release 1 mol of p-nitroaniline per second.

Hydrolysis of Casein with Alcalase, PepX and PepN

The prehydrolysis of casein was performed using Alcalase 2.4 L (Novozymes, Bagsvaerd, Denmark). The improved hydrolyses were performed with purified PepX and PepN. All experiments were repeated at least once, and the data reported were measured in triplicate. The error bars were not included in the graphical representations because the standard deviation was below 5%.

**Determination of the proteolytic enzymatic activity of Alcalase with o-phthalaldehyde (OPA).** The initial enzymatic activity of Alcalase 2.4 L was determined using casein (1%, w/v) in Na2HPO4/KH2PO4 buffer (50 mM; pH 7.0) as the substrate. The reaction contained 250 μL of the substrate and 25 μL of the diluted Alcalase 2.4 L solution, which was incubated at 37 °C and terminated at various time points with the addition of 25 μL trichloroacetic acid (TCA; 2 M). Following centrifugation (8,000 × g, 5 min), 25 μL of the solution was transferred to a microtiter plate, and 175 μL of the OPA reagent (22) was added while shaking for 5 sec. After exactly 2 min, the absorption was measured in an iEMS Reader (Labsystems, Helsinki, Finland) at 450 nm. The respective sample was mixed with TCA before adding the substrate for reference. One katal of proteolytic activity was defined as the amount of enzyme required to release 1 mol of serine equivalents per second.

**Prehydrolysis of casein with alcalase.** Casein (2.5%, w/v; 100 mL) was suspended in Na2HPO4/KH2PO4 buffer (50 mM; pH 7.0), and sodium azide (0.1%, w/v) was added. Alcalase (480 nkat mL solution−1) was added after preincubation of the solution (37 °C, 30 min). Samples (450 μL) were removed at various time points during hydrolysis until no increase in serine equivalents was observed (6.5 h). The samples were added to 50 μL of 3% SDS (10%, w/v) and heated to 80 °C for 10 min for enzyme inactivation. After centrifugation (8,000 × g, 5 min), the samples were analyzed using the OPA assay described above. The inactivated samples were stored at −20 °C for analyses conducted later. The hydrolyzed casein solution was heated to 75 °C for 30 min and stored at −20 °C for improved hydrolysis measurement.

**Improved hydrolysis with PepX and PepN.** The prehydrolyzed casein solution (see above) was used for improved casein hydrolysis (10 mL). Sodium azide (0.1%, w/v) was added to the prehydrolyzed casein solution and incubated at 37 °C for 30 min. PepX (50 nkat mL solution−1), PepN (50 nkat mL solution−1) or both enzymes (each 50 nkat mL solution−1) were added for further hydrolysis (10 h). Samples were removed at various time points during hydrolysis and were treated as described above.

**Results**

The original wild-type strain produced only low levels of PepX and PepN when cultivated in typical MRS medium (data not shown). Therefore, an efficient heterologous recombinant production system was established in *E. coli* to obtain high levels of both enzymes for more detailed biochemical and kinetic characterization.

**Sequencing of pepX and pepN from Lactobacillus helveticus ATCC 12046**

The PepN expression vector (pET-20b(+); pepN) was constructed, sequenced (Sequence S1) and used for expression in the *E. coli* BL21(DE3) strain. The nucleotide sequence of the pepX gene obtained was deposited in the GenBank database (accession number: JX826666) and exhibited 100% identity to that of *Lb. helveticus* CNRZ 32.

The PepN expression vector (pET-20b(+); pepN) was also constructed and sequenced (Sequence S2), and the gene sequence for pepN obtained was deposited in the GenBank database (accession number: JX826667). PepN exhibited 99% sequence identity to the corresponding gene of *Lb. helveticus* CNRZ 32.
Characterization of the Exopeptidases PepX & PepN

Heterologous Expression of PepX and PepN in E. coli

High levels of soluble PepX or PepN were individually expressed in E. coli BL21(DE3) using identical cultivation conditions (Fig. 1).

During the cultivation of the recombinant pET-20b(+)_pepX in E. coli BL21(DE3), the glucose was completely consumed after approx. 11 h when the cells entered the stationary phase (Fig. 1A). The OD_{600 nm} value for the cells increased up to 26 during cultivation (data not shown), and the maximum enzymatic activity of PepX was 800 μkatH-Ala-Pro-NA L^{-1}. The enzymatic activity was approximately constant over 11 h during the stationary phase. The wet cell biomass corresponded to 21 g L^{-1} (equal to approx. 6.5 g cdw L^{-1}).

An OD_{600 nm} value of 33 was observed for the production of recombinant PepN for the E. coli during cultivation (data not shown). The glucose was completely consumed by the cells after 14.5 h when entering the stationary phase (Fig. 1B). The maximum enzymatic activity of PepN was determined to be 1,000 μkatH-Ala-Pro-NA L^{-1}, which was nearly constant during the stationary phase. The wet cell biomass concentration at the end of the cultivation was 27 g L^{-1} (equal to approx. 8 g cdw L^{-1}).

Cells transformed with insert-free pET-20b(+) (reference) were cultivated under identical conditions to determine the background peptidase activities of the E. coli BL21(DE) strain. The background activities of the reference were approx. 0.025% for PepX and 1% for PepN.

Automated Purification of Recombinant PepX and PepN

Both His-tagged PepX and PepN were individually purified using an automated chromatographic procedure based on Ni-IMAC (see Figure S1 for the chromatogram). An enzymatic activity yield of 93% and a purification factor of 3.7 (specific activity: 967 nkatH-Ala-Pro-NA mg\text{protein}^{-1}) were obtained for PepX. The enzymatic activity yield for PepN was 78% after purification, with a purification factor of 2.9 (specific activity: 577 nkatH-Ala-Pro-NA mg\text{protein}^{-1}).

Molecular Mass Estimations

The SDS PAGE analysis of the peptidase samples, which were obtained from the cultures, indicated overexpressed protein bands at approx. 90 kDa for PepX and 95 kDa for PepN (data not shown). The SDS PAGE analyses of both purified enzymes after affinity chromatography and desalting are shown in Figure S2.

Native PAGE of the purified enzyme fractions, which were stained with Coomassie Brilliant Blue, and also for peptidase activity using the chromogenic substrates, resulted in single protein bands at approx. 180 kDa for PepX and 95 kDa for PepN (see Figure S2). These results suggest that the purified PepX was a homodimer with an apparent molecular mass of approx. 180 kDa, for which the theoretical calculation is 103.1 kDa (based on the amino acid sequence plus the His-tag). The purified PepN was a monomer with an apparent molecular mass of 95 kDa, for which the theoretical calculation is 96.9 kDa (based on the amino acid sequence plus the His-tag).

Characterization of PepX

pH and temperature optimum and thermal stability. PepX exhibited the highest activity in Na_{2}HPO_{4}/KH_{2}PO_{4} buffer at pH 6.5 (100%; Fig. 2A) whereas it exhibited higher activity in Bis-Tris/HCl buffer at pH 7.0 (91%) than at pH 6.5 (84%). The activity in Na_{2}HPO_{4}/KH_{2}PO_{4} buffer at pH 7.0 was only 87%. The highest activity of PepX was obtained at 50°C (100%; Fig. 2C), and it demonstrated a residual activity of only 8% at 65°C. PepX was preincubated at various temperatures over a period of 15 d to determine the thermal stability (Fig. 2E). The greatest thermal stability for the enzyme was observed at 0, 20 and 30°C, which resulted in more than 90% of the residual activity. Even at temperatures of 37 and 50°C, PepX exhibited residual activities of 79 and 34%, respectively. These results demonstrated the high thermal stability of this enzyme and its potential for application in industrial hydrolyses processes.

Kinetic studies. The kinetic experiments for PepX were conducted with the commercially available chromogenic substrates H-Ala-Pro-pNA, H-Arg-Pro-pNA and H-Gly-Pro-pNA-p-tosylate (Table 1). No substrate inhibition was observed in the concentration range tested (0.017–4.26 mM). The corresponding K_{m} and V_{max} values were calculated from Hanes plots. The K_{m} values determined were in the range between 1.3 and 11.3 mM. The highest relative catalytic efficiency (V_{max}/K_{m}) for PepX was obtained with the substrate H-Ala-Pro-pNA (100%), followed by H-Arg-Pro-pNA (59%) and H-Gly-Pro-pNA-p-tosylate (6%).
In addition, the potential inhibitory influence of PepX products (H-X-Pro-OH peptides) on the initial PepX activity was investigated. Therefore, the potential PepX products H-Gly-L-Pro-OH, H-L-Ala-L-Pro-OH, H-L-Arg-L-Pro-OH, H-L-Phe-L-Pro-OH, and H-L-Tyr-L-Pro-OH were added to the assays at final concentrations of 0.1, 1 or 10 mM. Inhibition was detected for the peptides containing an aromatic amino acid in their sequence. PepX was inhibited by 28% when H-L-Tyr-L-Pro-OH was present at a concentration of 10 mM. An even stronger inhibition of 38% was observed in the presence of 10 mM H-L-Phe-L-Pro-OH. A representative product inhibition is shown in Figure 3. Lineweaver-Burk linearization indicated that the inhibition was mixed-type (coincident noncompetitive and competitive inhibition). The inhibition constants were 30.1 mM for $K_i$, which is the binding constant for the enzyme, and 43.6 mM for $K_m$, which is the binding constant for the enzyme-substrate complex.

Effect of salts and metal ions on PepX activity. The influence of different mono- and divalent cations on PepX activity was extensively tested to characterize its behavior in a complex environment, such as food (Fig. 2G, Table 2). Concentrations of up to 300 mM of NaCl and KCl exerted a favorable effect on PepX activity (Fig. 2G). Conversely, PepX was inhibited by most divalent cations (Table 2). Nearly complete inhibition of the enzymatic activity was observed in the presence of 10 mM CuCl$_2$ or 0.0001 mM HgCl$_2$. No inhibition at low ZnCl$_2$ concentrations (0.1 and 1 mM) was observed. No inhibition for the monovalent cation NH$_4^+$ (NH$_4$Cl) was observed up to a concentration of 10 mM.

Effect of organic solvents, peptidase inhibitors, reducing agents, metal chelators, and denaturing agents on PepX activity. The substrates and the peptidase inhibitors were dissolved in organic solvents prior to their addition to the assay due to their limited solubility in pure water. The standard PepX assay, which used H-Ala-Pro-pNA as the substrate, contained 5.2% (v/v) DMF. In order to determine the individual influence of each organic solvent on PepX, the standard activity was measured in the presence of 10 mM CuCl$_2$ or 0.0001 mM HgCl$_2$. No inhibition at low ZnCl$_2$ concentrations (0.1 and 1 mM) was observed. No inhibition for the monovalent cation NH$_4^+$ (NH$_4$Cl) was observed up to a concentration of 10 mM.

| Substrate                  | $V_{max}$ [nkat mL$^{-1}$] | $K_m$ [mM] | $V_{max}/K_m$ [1 s$^{-1}$] | relative [%] |
|----------------------------|-----------------------------|------------|---------------------------|--------------|
| H-Ala-Pro-pNA (reference)$^2$ | 2309                        | 1.3        | 1.76                      | 100          |
| H-Arg-Pro-pNA              | 1549                        | 1.5        | 1.04                      | 59           |
| H-Gly-Pro-pNA-p-tosylate   | 1224                        | 11.3       | 0.11                      | 6            |

$^1$Protein concentration of the enzyme solution: 2.61 mg mL$^{-1}$.
$^2$Substrate for the standard assay. Triplicate measurements; the standard deviation was <5%.

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Characterization of PepN

**pH and temperature optimum and thermal stability.** The pH and temperature optimum of the purified PepN was investigated (Figs. 2B, 2D, 2F). PepN exhibited the highest activity in Na2HPO4/KH2PO4 buffer at pH 6.5 (100%). Bis-Tris/HCl buffer demonstrated a strong negative effect on the enzymatic activity (24% at pH 6.5; Fig. 2B). The reason for this effect is not clear.

Concerning the optimum assay temperature for PepN, the highest activity was observed at an unusually low temperature of 30°C (100%; Fig. 2D). A residual PepN activity of only 26% was measured at an assay temperature of 50°C. Concerning the thermal stability, incubation for 15 d resulted in residual PepN activities of 84% at 0°C, 61% at 30°C and 20% at 50°C (Fig. 2F). It is remarkable that PepN was not completely inactivated after incubation at 50°C for 15 d because of its low temperature optimum of 30°C. The inactivating effect of DMF, which was present in the standard assay used in the measurements of the optimal temperature, might explain the low optimal temperature. This result is consistent with the results below (see Table 2).

### Table 2. Effect of various solvents, cations, inhibitors, reducing agents, metal chelators, and denaturing agents on the activity of PepX and PepN from Lb. helveticus ATCC 12046 (at 37°C in 50 mM Na2HPO4/KH2PO4 buffer, pH 6.5).

| Substance | Concentration | Activity [%] | Activity [%] |
|-----------|---------------|--------------|--------------|
| Solvents [%(v/v)] |              | PepX | PepN | Solvents [%(v/v)] |              | PepX | PepN |
| Acetone   | 10            | 50   | 13   | DMSO      | 10            | 74   | 102  |
| EtOH      | 10            | 78   | 18   | DMF       | 10            | 37   | 23   |
| Cations [mM] |              | PepX | PepN | Cations [mM] |              | PepX | PepN |
| Ca2+      | 0.1           | 99   | 99   | Mg2+      | 0.1           | 90   | 96   |
|           | 1             | 89   | 65   | 1         | 89           | 92   |      |
|           | 10            | 59   | 8    | 10        | 90           | 58   |      |
| Co2+      | 0.1           | 99   | 186  | Mn2+      | 0.1           | 94   | 76   |
|           | 1             | 90   | 161  | 1         | 92           | 36   |      |
|           | 10            | 70   | 55   | 10        | 59           | 10   |      |
| Cu2+      | 0.001         | 96   | 54   | NH4+      | 0.1           | 99   | 95   |
|           | 0.01          | 89   | 39   | 0.1       | 99           | 96   |      |
|           | 0.1           | 34   | 20   | 10        | 100          | 94   |      |
|           | 1             | 21   | 2    | 100       | 93           | 89   |      |
|           | 10            | 1    | 0    | 1         | 95           | 82   |      |
| Fe2+      | 0.1           | 91   | 79   | 10        | 20           | 21   |      |
|           | 1             | 58   | 6    | 125       | 78           |      |      |
|           | 10            | 37   | 4    | 137       | 34           |      |      |
| Hg2+      | 0.0001        | 6    | 94   | 10        | 36           | 13   |      |
|           | 0.001         | 3    | 2    | 3         | 2            |      |      |
|           | 0.01          | 0.2  | 0    | 0         | 0            |      |      |
| Reagents [mM] |              | PepX | PepN | Reagents [mM] |              | PepX | PepN |
| β-mercaptoethanol | 0.1          | 108  | 74   | Pepstatin A | 0.001         | 97   | 95   |
|           | 1             | 104  | 64   | 0.01      | 92           | 90   |      |
|           | 10            | 105  | 39   | 0.1       | 87           | 86   |      |
| DTT2      | 0.1           | 100  | 110  | 1,10-phenanthroline4 | 0.001 | n.d. | 102  |
|           | 1             | 106  | 86   | 0.01      | n.d.         | 21   |      |
|           | 10            | 105  | 29   | 0.1       | 100          | 0    |      |
| EDTA2     | 0.000001      | n.d. | 86   | 1         | 100          | 0    |      |
|           | 0.00001       | n.d. | 75   | 10        | 100          | 0    |      |
|           | 0.001         | n.d. | 44   | 0.1       | 96           | 102  |      |
|           | 0.0001        | n.d. | 8    | 1         | 66           | 85   |      |
|           | 0.01          | n.d. | 1    | 10        | 11           | 74   |      |
|           | 0.1           | 104  | 0    | SDS2      | 0.1          | 106  | 80   |
|           | 1             | 104  | 0    | 1         | 84           | 0    |      |
|           | 10            | 113  | 0    | 10        | 0            | 0    |      |
| Imidazole2 | 0.1           | 97   | 84   | Urea2     | 0.1          | 101  | 99   |
|           | 1             | 94   | 86   | 1         | 100          | 94   |      |
|           | 10            | 92   | 87   | 10        | 102          | 90   |      |

1The value of 100% was determined in the presence of the corresponding solvent without the additional substance. The substance was dissolved in: H2O; DMSO; Acetone; EtOH. Triplicate measurements; the standard deviation was <5%; n.d., not determined.

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which a strong PepN sensitivity to DMF was observed (the addition of 10% (v/v) DMF resulted in 23% residual activity).

**Kinetic studies.** The kinetic parameters for PepN using different chromogenic substrates are summarized in Table 3. PepN demonstrated broad substrate specificity, which included hydrophobic, aromatic, acidic, and basic amino acids. A PepN peptidase activity was detected for H-Glu-NA and H-His-NA (data not shown), but due to the poor solubility properties of these substrates, a determination of the kinetic constants was not possible. The kinetic parameters of the soluble substrates were determined by both Michaelis-Menten and Lineweaver-Burk analyses, because all substrates demonstrated clear substrate inhibition at higher concentrations. Representative kinetic analyses determined by both Michaelis-Menten and Lineweaver-Burk linearization was always higher than those determined by Michaelis-Menten analysis, due to the linearization and the substrate inhibition. However, Michaelis-Menten analysis was more suitable for determining the inhibition constants (K_{IS}) for the enzyme (the substrate concentration at half V_max inhibition).

High V_max values were measured using the substrates H-Lys-NA and H-Arg-NA followed by H-Ala-NA and H-Leu-NA. The lowest K_m value was obtained using the substrate H-Lys-NA, which also led to the best hydrolytic efficiency (V_max/K_m) of 11.4 s^{-1}. This is the first time, to the best of our knowledge, that a strong PepN sensitivity to DMF was observed (the addition of 10% (v/v) DMF resulted in 23% residual activity).

A selection of various free amino acids were added to the standard assay to additionally determine the potential product inhibition of PepN (Table 4). Significant product inhibition was observed using the amino acids L-Phe (10 mM; 24% residual activity) and L-Arg (10 mM; 19% residual activity); only a slight inhibition was observed for L-Ala and Gly, and L-Pro and L-Asp did not inhibit PepN within the concentration range tested. Therefore, product inhibition was not as general as substrate inhibition, but clearly occurred in some cases.

**Effect of salts and metal ions on PepN activity.** PepN activity was also measured in the presence of salts and metal ions to gain insights into a potential industrial application in complex matrices. PepN activity decreased at low NaCl or KCl concentrations (Fig. 2H) and was also inhibited by several divalent cations (Table 2). Here, PepN was strongly inhibited by Cu^{2+} (1 mM; 2% residual activity), Fe^{2+} (1 mM; 6% residual activity) and Hg^{2+} (0.001 mM; 2% residual activity). Almost no inhibition was observed for the monovalent cation NH_4^+ (NH_4Cl) at concentrations up to 10 mM. The divalent cation Ca^{2+} demonstrated an activating effect on PepN activity (186–161%) at lower concentrations (0.1–1 mM).

PepN is known to be a neutral zinc metallopeptidase [23,24]. We prepared the apo-enzyme (apo-PepN) and investigated the reactivating effect of divalent metal ions. Apo-PepN was incubated with different concentrations of divalent cations (Fig. 5). Co^{2+} was found to enhance PepN activity to 180% at a concentration of 0.25 mM CoCl_2. And even higher concentrations of the latter, up to 1 mM, preserved this high activity. The “natural” Zn^{2+} ion at a concentration of 0.1 mM restored the PepN activity to 99% compared with the activity of native holo-PepN. However, higher concentrations of ZnCl_2 decreased the PepN activity, which was consistent with the inhibition studies (Table 2). Only slightly lower recovery yields of up to 94% were obtained with MnCl_2 in the broad range of 0.1–1 mM.

These results suggest that a replacement of the native Zn^{2+} ion of PepN by a Co^{2+} ion would be useful for a potential biotechnological application of PepN due to the significant increase of its catalytic power.

**Effects of organic solvents, peptidase inhibitors, reducing agents, metal chelators, and denaturing agents on the enzymatic activity.** Similar to PepX, the influence of various agents on the enzymatic activity of PepN were investigated. The results are summarized in Table 2 and similar calculations were performed, as previously described. The enzymatic activity of PepN was strongly reduced by the addition of 10% (v/v) acetone, ethanol and DMF to 13–23% of the reference activity. It was surprising that the addition of DMSO did not influence the PepN activity (102%). An insignificant inhibition was observed using the

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**Figure 4. Determination of the kinetic parameters of PepN for the substrate H-Ala-NA according to Michaelis-Menten analysis (A) and Lineweaver-Burk linearization (B) (each point represents the average of triplicate measurements; the standard deviation was <5%).**

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Improved Casein Hydrolysis Using PepX and PepN

The performance of both purified enzymes (PepX, PepN) in milk protein hydrolysis was investigated as follows. Solubilized casein was prehydrolyzed with the commercial endopeptidase preparation Alcalase 2.4 L (480 nkat mL\(^{-1}\)) at 37°C over a period of 6.5 h (data not shown). The Alcalase was then inactivated by heat treatment prior to the assay. This prehydrolysis step was necessary because PepX and PepN are exopeptidases, as previously mentioned. PepX and PepN were added individually or in combination to the prehydrolyzed samples (Fig. 6). The highest increase in the \(rDH\) was obtained using a combination of PepX and PepN (approx. 132%), which demonstrates the synergistic action of both lactobacilli peptidases. PepX alone increased the \(rDH\) only very slightly (approx. 12%) due to its limited substrate specificity (cleavage of X-Pro-peptides at the N-terminus, where X is any amino acid). PepN alone strongly increased the \(rDH\) (approx. 100%) due to its very broad substrate specificity, in which all amino acids can be cleaved from the N-terminus until X-Pro-bonds are essential for the activity of this enzyme. As expected, the anionic surfactant SDS (1 mM) completely inactivated PepN.

### Table 3. Kinetic parameters of PepN\(^1\) using chromogenic \(p\)-nitroaniline substrates.

| Substrate          | \(V_{max}\) [nkat mL\(^{-1}\)] | \(K_m\) [mM] | \(V_{max}/K_m\) [1 s\(^{-1}\)] | \(K_{mS}\) [mM] |
|--------------------|--------------------------------|--------------|--------------------------------|---------------|
| H-Ala-pNA (reference)\(^2\) | 769                           | 0.43         | 5.72                           | 1619          |
| H-Lys-pNA - 2HBr    | 1552                          | 0.14         | 9.91                           | 2068          |
| H-Arg-pNA - 2HCl    | 1742                          | 0.14         | 4.98                           | 3619          |
| H-Leu-pNA           | 851                           | 0.17         | 2.92                           | 1240          |
| H-Phe-pNA\(^3\)     | 254                           | 0.15         | 1.87                           | 385           |
| H-Val-pNA           | 30.7                          | 0.27         | 3.41                           | 49.8          |
| H-Ile-pNA           | 10.1                          | 0.17         | 2.17                           | 17.4          |
| H-Pro-pNA           | 19.9                          | 0.94         | 7.15                           | 53.5          |
| H-Gly-pNA           | 15.8                          | 0.69         | 8.87                           | 37.8          |

\(1\) Protein concentration of the enzyme solution: 2.16 mg mL\(^{-1}\).

\(2\) Substrate for the standard assay.

\(3\) The maximum substrate solubility was 3.73 mM under the assay conditions. Triplicate measurements; the standard deviation was <3%.

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Table 4. Relative PepN activities (%) for the standard assay (substrate: H-Ala-pNA) in the presence of various potential product inhibitors.

| Potential product inhibitors | Concentration [mM] |
|------------------------------|--------------------|
| L-Ala                        | 0.1 | 0.2 | 0.9 |
| L-Arg                        | 0.1 | 69  | 19  |
| L-Asp                        | 0.1 | 99  | 89  |
| Gly                          | 0.1 | 99  | 92  |
| L-Phe                        | 0.2 | 75  | 24  |
| L-Pro                        | 0.2 | 100 | 13  |

Triplicate measurements; the standard deviation was <3%.

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### Table 4.

| Substance | Michaelis-Menten | Lineweaver-Burk | Catalytic efficiency |
|-----------|-----------------|-----------------|---------------------|
|           | \(V_{max}\)     | \(K_m\)         | \(K_{m relative}\) |
|           | [nkat mL\(^{-1}\)] | [mM]            | [%]                |
| H-Ala-pNA | 769             | 0.43            | 5.72               | 1619 | 1.43 | 1.13 | 9.91 |
| H-Lys-pNA - 2HBr | 1552 | 0.14 | 9.91 | 2068 | 0.18 | 11.40 | 100 |
| H-Arg-pNA - 2HCl | 1742 | 0.14 | 4.98 | 3619 | 0.42 | 8.62 | 75.6 |
| H-Leu-pNA | 851             | 0.17            | 2.92               | 1240 | 0.29 | 4.28 | 37.5 |
| H-Phe-pNA\(^3\) | 254 | 0.15 | 1.87 | 385 | 0.24 | 1.60 | 14.0 |
| H-Val-pNA | 30.7            | 0.27            | 3.41               | 49.8 | 0.63 | 0.08 | 0.70 |
| H-Ile-pNA | 10.1            | 0.17            | 2.17               | 17.4 | 0.38 | 0.05 | 0.44 |
| H-Pro-pNA | 19.9            | 0.94            | 7.15               | 53.5 | 3.71 | 0.01 | 0.13 |
| H-Gly-pNA | 15.8            | 0.69            | 8.87               | 37.8 | 2.70 | 0.01 | 0.13 |

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**Discussion**

In the current study, PepX (X-prolyl dipeptidyl aminopeptidase; EC 3.4.14.11) and PepN (aminopeptidase N; EC 3.4.11.2) from *Lactobacillus helveticus* ATCC 12046 were cloned and expressed in *E. coli*. The enzymes were purified, characterized and applied in casein hydrolysis.

### Recombinant production of PepX and PepN.

The maximum enzymatic activity of PepX was 800 \(\mu\)katH-Ala-Pro-pNA L\(^{-1}\) in the current study. Vesanto and colleagues [25] obtained a PepX activity of only 4.1 \(\mu\)katH-Ala-Pro-pNA L\(^{-1}\) for the recombinant PepX from *Lb. helveticus* 53/7; they used the *E. coli* JM105 strain for expression and pKK223-3 as the expression vector, which are not as suitable for the production of the enzyme as our host strain. An activity of 1,000 \(\mu\)katH-Ala-pNA L\(^{-1}\) was determined for the recombinant PepN. The expression of PepN from *Lb. helveticus* WSU19 using *E. coli* DH5\(\alpha\) has been published previously [26]. However, the authors did not report the enzymatic activity obtained. The recombinant expression of PepN was also obtained in *Lactobacillus lactis* [27], but comparable activity data were not provided by the authors.

### Molecular mass estimation of PepX and PepN.

The molecular masses of PepX and PepN were estimated by PAGE after automated purification. PepX from *Lb. helveticus* ATCC 12046 appeared as a homodimer on native PAGE with an estimated molecular mass of 180 kDa. Dimeric forms of PepX were also described from *Lb. helveticus* ATCC 57/3 [25] and IFO3809 [28]. In contrast, monomeric PepX from *Lb. helveticus* ITG LH1 [5] and CNRZ 32 [11,29] have also been reported previously. The
PepN from *Lb. helveticus* ATCC 12046 was a monomeric enzyme with an apparent molecular mass of 95 kDa, which is consistent with the monomeric PepN described for *Lb. helveticus* SBT 2171 [8] and CNRZ 32 [7]. Additionally, a trimeric form of an aminopeptidase with a molecular mass of approx. 129 kDa (43 kDa per subunit) was described for *Lb. helveticus* JCM 1004 [30].

**Characterization of PepX.** The optimal pH for PepX from *Lb. helveticus* ATCC 12046 was 6.5, which is identical to that reported for PepX from *Lb. helveticus* LHE-511 [12]. However, the PepX from *Lb. helveticus* ITG LH1 and CNRZ 32 demonstrated a slightly higher pH optimum of 7.0 [5,11]. The temperature optimum of PepX from *Lb. helveticus* ATCC 12046 was determined to be 50°C, which is similar to that of the PepX from *Lb. helveticus* LHE-511 [12]. A lower optimal temperature of 40°C was measured for the PepX enzymes isolated from *Lb. helveticus* ITG LH1 [5] and CNRZ 32 [11]. The PepX from *Lb. helveticus* ATCC 12046 demonstrated a residual activity of 34% after incubation for 15 d at 50°C. In comparison, the PepX from *Lb. helveticus* ITG LH1 lost approx. 40% of its activity after 30 minutes at 50°C [5].

The kinetic parameters of PepX from *Lb. helveticus* ATCC 12046 were determined using three chromogenic peptides (H-Ala-Pro-pNA, H-Arg-Pro-pNA and H-Gly-Pro-pNA). The *Km* values ranged between 1.3 and 11.3 mM. These values were approx. 20-fold higher than those reported for the PepX from *Lb. helveticus* ITG LH1 [5]. The relative activity of PepX from *Lb. helveticus* ATCC 12046 for the three different substrates was determined in the order: H-Ala-Pro-pNA>H-Arg-Pro-pNA>H-Gly-Pro-pNA. This result is consistent with the results presented by Degraeve and Martial-Gros [5], as well as by Miyakawa *et al.* [12] for PepX from other *Lb. helveticus* strains. The exopeptidase PepX of the current study was product inhibited by some dipeptides that contained aromatic amino acids. This type of inhibition has not been described for other exopeptidases. However, some commercial preparations also demonstrated product inhibition by endopeptidases during food protein hydrolysis [13,14,31].

The influence of various mono- and divalent cations on the PepX activity of *Lb. helveticus* ATCC 12046 was investigated. A slight activating effect of NaCl and KCl was also observed for PepX from *Lb. helveticus* ITG LH1 [5] and CNRZ 32 [11]. The PepX from *Lb. helveticus* LHE-511 was not inhibited by 1 mM FeCl2 [12], whereas the PepX from *Lb. helveticus* ITG LH1 [5] and the PepX in this study were strongly inhibited. In addition, the PepX activity determined in this study and from *Lb. helveticus* LHE-511 [12] increased in the presence of 1 mM ZnCl2 to 137 and 111%, respectively. At an identical ZnCl2 concentration, the PepX activity from *Lb. helveticus* ITG LH1 [5] and CNRZ 32 [11] decreased to approx. 8 and 40% residual activity, respectively.
In our studies, PepX was inhibited by PMSF and was most likely a serine peptidase. This finding is consistent with previous reports. Residual activities of 66, 38, 34, and 46% were determined in the presence of 1 mM PMSF for PepX from the strains *Lb. helveticus* ATCC 12046 (the current study), ITG LH1 [5], LHE-511 [12], and CNRZ 32 [11], respectively. These results suggest the involvement of a serine residue in catalysis. Moreover, the PepX from *Lactococcus lactis* was described as a serine peptidase [32].

The reducing agents DTT and β-mercaptoethanol did not demonstrate an inactivating effect on PepX from *Lb. helveticus* ATCC 12046, which is similar to the results described for PepX from *Lb. helveticus* ITG LH1 [5] and LHE-511 [12].

The PepX from *Lb. helveticus* LHE-511 [10] and CNRZ 32 [11] were inhibited in the presence of metal-complexing agents, such as EDTA and 1,10-phenanthroline, thus indicating a metal ion dependency. This dependency was not observed in the current study or with the PepX from *Lb. helveticus* ITG LH1 [5]. In conclusion, the PepX described currently possesses clearly unique features and shares some characteristics of PepX peptidases described previously. Product inhibition has been demonstrated thus far only for the PepX described in this report.

**Characterization of PepN.** The PepN from *Lb. helveticus* ATCC 12046 demonstrated an identical pH optimum at 6.5 to the PepN from CNRZ 32 [7] and ITG11 [33]. The PepN from *Lb. helveticus* LHE-511 [10] and JCM 1004 [30] exhibited a maximal activity at pH 7.0.

The PepN from *Lb. helveticus* ATCC 12046 demonstrated the highest activity at 30°C. Other PepNs demonstrated higher temperature optima: 37°C for the *Lb. helveticus* strain LHE-511 [10], 40°C for the JCM 1004 strain [30] and 45°C for the CNRZ 32 strain [7]. It was surprising that PepN from *Lb. helveticus* ATCC 12046 exhibited remarkable thermal stability, demonstrating 20% residual activity after 15 d of incubation at 50°C. In comparison, the PepN from *Lb. helveticus* JCM 1004 demonstrated only 5% residual activity after only 30 min of incubation at 50°C [30]. A similar weak thermal stability of approx. 30% residual activity was reported for the PepN from *Lb. helveticus* LHE-511 after 2 h of incubation at 50°C [10].

The kinetic parameters of PepN from *Lb. helveticus* ATCC 12046 were determined using nine commercially available chromogenic peptides. The relative activities measured demonstrated an identical order (H-Lys-pNA>H-Arg-pNA>H-Leu-pNA>H-Ala-pNA) to other PepN peptidases from *Lb. helveticus* LHE-511 [10], CNRZ 32 [7] and JCM 1004 [30]. It was remarkable that PepN from *Lb. helveticus* ATCC 12046 was able to hydrolyze H-Pro-pNA, which has only been reported for the PepN of *Lb. helveticus* SBT 2171 [8] thus far. Other PepNs (from *Lb. helveticus* LHE-511 [10], CNRZ 32 [7] and *Lb. curvatus* DPC2024 [34]) could not hydrolyze this substrate.

The substrate inhibition observed for the PepN from *Lb. helveticus* ATCC 12046 is reported for the first time. This information is valuable for potential biotechnological applications of the enzyme in the future, for example, in fed-batch biotransformation processes avoiding high substrate concentrations.

The addition of NaCl or KCl displayed a negative effect on the PepN activity in this study. These results are consistent with results for PepN from *Lb. helveticus* CNRZ 32 published previously [7]. The activity of PepN from *Lb. helveticus* ATCC 12046 and PepN from *Lb. helveticus* JCM 1004 [30] was reduced after the addition of 1 mM FeCl₂ to 6 and 45.7% residual activity, respectively. A similar result (19% residual activity) was reported for PepN from *Lb. helveticus* LHE-511 [10]. Additionally, Cu²⁺ ions (1 mM) decreased the PepN activity of this study, *Lb. helveticus* JCM 1004 [30], *Lb. helveticus* CNRZ 32 [7], and *Lb. helveticus* LHE-511 [10] to 2, 71.7, 0, and 4% residual activity, respectively. The heavy metal salt HgCl₂ (0.001 mM) strongly inactivated PepN (2% residual activity). The PepN from *Lb. helveticus* CNRZ 32 demonstrated more tolerance (10% residual activity at 100-fold higher 0.1 mM HgCl₂) [7]. The addition of CoCl₂ (1 mM) increased the activity of PepN (161%) in this study, as similarly reported for the PepN from *Lb. helveticus* JCM 1004 [30], CNRZ 32 [7] and LHE-511 [10], for which the activation reached 151, 179, and 181%, respectively. The most efficient reactivation of apo-PepN was observed using 0.25 mM CoCl₂ (180% activity) in this study. Moreover, the activity of apo-PepN from *Lb. helveticus* SBT 2171 was restored up to 160% using 100 μM CoCl₂ [9].

The strong inhibition of PepN in this study by metal-complexing reagents, such as EDTA and 1,10-phenanthroline, indicated that the enzyme is a metallopeptidase, as also described for other PepNs [7,10].

**Improved casein hydrolysis.** The aminopeptidases PepX and PepN were applied to a prehydrolyzed casein solution because there are no endopeptidases that alone provide a high degree of protein hydrolysis [35]. The combined use of PepX and PepN increased the rDH by a remarkable 132% due to the complementary substrate specificities of both peptidases. The general potential of PepX combined with PepN from *Lactococcus lactis* ssp. cremoris AM2 in the enhancement of the enzymatic degradation of chemically synthesized peptides has been demonstrated [36]. The debittering effect of a tryptic digest of β-casein with porcine kidney general aminopeptidase and X-prolyl dipeptidyl aminopeptidase from *Lc. lactis* ssp. cremoris AM2 was described [37].

A limited productivity of the peptidases is assumed in enzymatic protein hydrolysis using a batch process due to potential substrate or product inhibition. These types of inhibition were recognized for PepX and PepN in our study. Consequently, a continuous process, through the use of an enzymatic membrane reactor (EMR), would overcome these limitations, as was recently demonstrated in the casein hydrolysis using the commercial pepidase preparation BLAP (*Bacillus lichenoides* alkaline peptidases) [31], whereby the inhibiting peptides produced were continuously separated from the enzymes by ultrafiltration membranes. In such a stable, enzyme-rich, such as the PepX and PepN described, might be used for a long operating time at low substrate and product concentrations. In addition, a standardized product quality of the protein hydrolysate with no bitter taste and no allergenic effects would be possible in the future.

**Conclusion**

Recombinant production of the exopeptidases PepX and PepN from *Lb. helveticus* ATCC 12046 in *E. coli* resulted in high levels of soluble enzymes. Both peptidases were easily purified using His-tag affinity chromatography (IMAC) and featured a reasonable thermostability at 50°C for 15 d. The kinetic investigations of both purified peptidases demonstrated product inhibition. Substrate inhibition was also observed for PepN. PepN possessed a broad substrate specificity in which even H-Pro-pNA was hydrolyzed. The synergistic effect of PepX and PepN resulted in a significantly higher degree of casein hydrolysis when used in combination. The properties of both enzymes have the potential for future applications in food protein hydrolysis using an enzyme membrane reactor.
Supporting Information

Figure S1 Purification chromatogram of PepX. This file contains the chromatogram of the automated purification of PepX.
(PDF)

Figure S2 SDS- and native PAGE. This file contains the SDS and native PAGE analyses for recombinant PepX and PepN during purification.
(PDF)

Sequence S1 Sequences of pepX/PepX. This file contains the nucleotide sequence of the pepX gene and the translated amino acid sequence from Lactobacillus helveticus ATCC 12046.
(PDF)

Sequence S2 Sequences of pepN/PepN. This file contains the nucleotide sequence of the pepN gene and the translated amino acid sequence from Lactobacillus helveticus ATCC 12046.
(PDF)

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Author Contributions

Conceived and designed the experiments: TS TE LF. Performed the experiments: TS TE MS. Analyzed the data: TS TE MS SLW LF. Contributed reagents/materials/analysis tools: TS TE MS SLW LF. Wrote the paper: TS TE LF.

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