

A fusion protein consisting of the exopeptidases PepN and PepX—production, characterization, and application

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Abstract Nowadays, general and specific aminopeptidases are of great interest, especially for protein hydrolysis in the food industry. As shown previously, it is confirmed that the general aminopeptidase N (PepN; EC 3.4.11.2) and the proline-specific peptidase PepX (EC 3.4.14.11) from *Lactobacillus helveticus* ATCC 12046 show a synergistic effect during protein hydrolysis which results in high degrees of hydrolysis and reduced bitterness. To combine both activities, the enzymes were linked and a fusion protein called PepN-L1-PepX (FUS-PepN-PepX) was created. After production and purification, the fusion protein was characterized. Some of its biochemical characteristics were altered in favor for an application compared to the single enzymes. As an example, the optimum temperature for the PepN activity increased from 30 °C for the single enzyme to 35 °C for FUS-PepN. In addition, the temperature stability of PepX was higher for FUS-PepX than for the single enzyme (50 % compared to 40 % residual activity at 50 °C after 14 days, respectively). In addition, the disulfide bridge-reducing reagent β -mercaptoethanol did not longer inactivate the FUS-PepN activity. Furthermore, the K_M values decreased for both enzyme activities in the fusion protein. Finally, it was found that the synergistic hydrolysis performance in a casein hydrolysis was not reduced for the fusion protein. The increase of the relative degree of

hydrolysis of a prehydrolyzed casein solution was the same as it was for the single enzymes. As a benefit, the resulting hydrolysate showed a strong antioxidative capacity (ABTS-IC₅₀ value: 5.81 $\mu\text{g mL}^{-1}$).

Keywords *Lactobacillus helveticus* · Fusion protein · PepX · PepN · Casein hydrolysis · Antioxidative activity

Introduction

The usage of peptidases had a long history in the food industry (Stressler et al. 2015). They have been used, for example, in cheesemaking, baking, and meat tenderization (Rao et al. 1998). In addition, peptidases are used to produce protein hydrolysates, which can be applied for emulsification, gelatinization, or as seasoning (Sprössler 2003). The hydrolysis of food proteins can generally result in improved digestibility, modification of sensory quality, such as texture or taste, improvement of antioxidant capability, and reduction in allergenic compounds (Tavano 2013). A potential disadvantage of enzymatic hydrolysis of proteins is that the resulting hydrolysates can have a bitter taste caused by peptides (<10 kDa), which are composed mainly of hydrophobic amino acids (e.g., Leu, Ile, Pro; Saha and Hayashi 2001). The usage of exopeptidases can result in a reduction of bitterness of the hydrolysate due to an increased degree of hydrolysis (FitzGerald and O’Cuinn 2006; Nishiwaki et al. 2002; Raksakulthai and Haard 2003; Saha and Hayashi 2001; Tan et al. 1993). Possible sources of these exopeptidases are lactic acid bacteria (LAB). In general, LAB are Gram-positive, aerobic to facultative anaerobic, asporogenous rods and cocci, which are oxidase-, catalase-, and benzidine-negative (Carr et al. 2002). Different genera of LAB are *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus*, *Oenococcus*,

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Enterococcus, and *Leuconostoc* (Carr et al. 2002). Due to the fact that LAB are nonpathogenic organisms with the generally recognized as safe (GRAS) status (Mayo et al. 2010), they can be used in the production of different fermented food products. *Lactobacillus helveticus*, for instance, is one of the most often used species of LAB for the production of fermented milk beverages and some types of hard cheese (Griffiths and Tellez 2013). Due to the fact that *Lb. helveticus* is auxotrophic for multiple amino acids (Hebert et al. 2000), it has a very strong proteolytic system to obtain all its necessary amino acid requirements (Griffiths and Tellez 2013). The proteolytic system of LAB can generally be divided into three groups based on their function: the extracellular peptidases, different types of transporters, and intracellular peptidases (Kunji 1996; Mayo et al. 2010). Firstly, the extracellular peptidases break proteins down into peptides. Secondly, these products when broken down will be translocated across the cytoplasm membrane by the transport system. Thirdly, the intracellular peptidases degrade the translocated peptides into smaller peptides and amino acids. These intracellular peptidases have distinct but overlapping activity (Kunji 1996) and can be further divided into endopeptidases and exopeptidases. Based on their specificity, the exopeptidases can be differentiated, for example, into the general aminopeptidase PepN (EC 3.4.11.2) (Stressler et al. 2013b), the proline-specific peptidases such as PepX (EC 3.4.14.11) (Stressler et al. 2013b; Stressler et al. 2014) and PepP (EC 3.4.11.9) (Stressler et al. 2012), or the glutamyl (aspartyl)-specific aminopeptidase PepA (EC 3.4.11.7) (Exterkate and De Veer 1987; Stressler et al. 2016). The usage of a proline-specific X-prolyl-dipeptidyl aminopeptidase (PepX; EC 3.4.14.11) in combination with a general aminopeptidase N (PepN; EC 3.4.11.2) on a prehydrolyzed casein solution results in a reduction of bitterness of the hydrolysate (FitzGerald and O’Cuinn 2006). In previous studies, our group produced the exopeptidases PepX and PepN from *Lb. helveticus* ATCC 12046 recombinantly in *Escherichia coli*. It was shown that the synergistic effect of these two peptidases results in an increase of the relative degree of hydrolysis of a prehydrolyzed casein solution by approximately 132 % (Stressler et al. 2013b). In addition, the bitterness of the hydrolysate decreased (unpublished data). This hydrolysis was performed in a discontinuous batch process, as it is mainly performed in industrial biotransformations nowadays (Merz et al. 2015b; Rios et al. 2004). An alternative to discontinuous batch processes is continuous biotransformation using an enzyme membrane reactor (Giorno and Drioli 2000; Merz et al. 2015b). This approach has the great advantage that the enzymes can be reused and, thus, the process is more economical (Brena et al. 2013; Merz et al. 2015b). However, the process has to ascertain that the enzymes do not penetrate the membrane. A possible way to ensure this is to increase the molecular mass of the enzymes used in the enzyme membrane reactor (EMR) process. This

can be realized by the preparation of so-called cross-linked enzyme aggregates (CLEA). Here, the enzymes will be precipitated, for example, by the addition of salts or organic solvents (Hofland et al. 2000) and, subsequently, cross-linked by glutaraldehyde (Cao et al. 2001). In a recently published study by our group, combi-CLEAs of PepN and PepX were prepared and used for casein hydrolysis (Stressler et al. 2015). The study showed that the relative degree of hydrolysis of the prehydrolyzed casein solution was increased, but only by about 52 % (Stressler et al. 2015) and not by about 132 % as it was for the free enzymes (Stressler et al. 2013b). This difference was explained by the accessibility of the casein-derived peptides to the active site. Only short peptides can enter the active site, and therefore, fewer substrates were present for the combi-CLEAs (Stressler et al. 2015). So-called fusion proteins can be produced as a second method to increase the molecular mass. Here, the gene sequences of, for example, two enzymes can be linked. As a second advantage, the protein produced should have both enzyme activities desired (Hong et al. 2006; Lu and Feng 2008; Trujillo et al. 1997). However, the biochemical characteristics of the single enzymes and the fusion enzymes can be different. The fusion enzymes, for example, can have either no enzyme activity, lesser, or even higher activity (Lu and Feng 2008). The direct fusion of functional domains without a linker may lead to many undesirable outcomes (Chen et al. 2013), including misfolding of the fusion proteins (Zhao et al. 2008), low yield in protein production (Amet et al. 2009), or impaired bioactivity (Bai et al. 2005; Bai and Shen 2006). Many different linkers can be applied for the production of fusion proteins (Chen et al. 2013). In the current study, the ten-amino acid linker SSGLVPRGSH (L1) was used, as it is a standard feature in pET vectors to fuse the target protein with a His-tag (Kadow et al. 2014). To the best of our knowledge, no fusion proteins have yet been produced for exopeptidases. Thus, the aim of the current study was the cloning and heterologous recombinant production of a fusion protein consisting of the exopeptidases PepN and PepX from *Lb. helveticus* ATCC 12046 and its biochemical characterization. Furthermore, the fusion protein was applied for casein hydrolysis, and the antioxidative capacity of the resulting hydrolysate was investigated.

Materials and methods

Chemicals, enzymes, kits, materials, and devices

All chemicals were of analytical grade and purchased from Sigma-Aldrich (Taufkirchen, Germany), Carl Roth GmbH (Karlsruhe, Germany), or Applichem (Darmstadt, Germany). Hexokinase/glucose-6-phosphate dehydrogenase was purchased from Megazyme International Ireland (Wicklow,

Ireland) and was used for the glucose concentration determination assay based on the commercial D-glucose/D-fructose test kit from R-Biopharm AG (Darmstadt, Germany; product code 10 139 106 035). Chromogenic peptides were obtained from Bachem AG (Bubendorf, Switzerland). Molecular mass markers were bought from Thermo Scientific (Schwerte, Germany), SERVA Electrophoresis GmbH (Heidelberg, Germany), and GE Healthcare (München, Germany). The enzymes required for molecular biological work were purchased from NEB (Frankfurt, Germany), Qiagen (Hilden, Germany), Thermo Scientific (Schwerte, Germany), or Roche Applied Science (Penzberg, Germany). Kits for molecular biological work were obtained from Thermo Scientific (Schwerte, Germany) or Qiagen (Hilden, Germany). Agarose was bought from SERVA Electrophoresis GmbH (Heidelberg, Germany). PD-10 columns were obtained from GE Healthcare (München, Germany). The bioreactor cultivation was realized using the Minifors system (Infors AG, Bottmingen/Basel, Switzerland). The MINI-PROTEAN system (Bio-Rad Laboratories GmbH, München, Germany) was used for polyacrylamide gel electrophoresis. The ÄKTA-FPLC system (GE Healthcare, München, Germany) was used for protein purification.

Bacterial strains

Escherichia coli XL1 Blue (Merck, Darmstadt, Germany) and *E. coli* BL21(DE3) (Novagen, Madison, USA) were used as the hosts for cloned polymerase chain reaction (PCR) products and T7 expression work, respectively. Standard protocols were employed for the preparation and transformation of competent *E. coli* cells with plasmid DNA via heat shock (Sambrook and Russell 2001). The *E. coli* cells were cultivated as described previously (Stressler et al. 2012; Stressler et al. 2013b).

Cloning, construction of expression vector, and sequencing

PCR was performed using HotStar HiFidelity polymerase (Qiagen), according to the manufacturer's instructions. All amplification primers (see Supplemental Material Table S1) used in this study were synthesized by biomers.net GmbH (Ulm, Germany). Standard techniques in recombinant DNA were employed for the experiments (Sambrook and Russell 2001). The plasmids containing the genes of *pepX* (pET20b(+)_{*pepX*}) and *pepN* (pET20b(+)_{*pepN*}) from *Lb. helveticus* ATCC 12046, respectively, were constructed in a previously published study (Stressler et al. 2013b) and stored in *E. coli* XL1 Blue cells. The plasmids were isolated using the GeneJET Plasmid Miniprep kit (Fermentas), according to the manufacturer's instructions, before constructing the fusion gene by overlap extension PCR. The purified plasmids were

used as a template to amplify *pepN_L1* (primers 1 and 2; see Supplemental Material Table S1) and *L1_pepX* (primers 3 and 4; see Supplemental Material Table S1). The PCR products resulted in sticky ends containing the linker sequence. They were purified using the QIAquick PCR Purification kit (Qiagen), according to the manufacturer's instructions. Subsequently, the sticky ends were assembled in an overlap extension PCR to create the fusion gene *pepN_L1_pepX*. Therefore, the purified PCR products and the primers 1 and 4 (see Supplemental Material Table S1; both were added to increase the yield of correct fusion gene) were used in a next PCR reaction with the following program: 95 °C for 5 min, then 16 cycles of 95 °C for 15 s, 68 °C (−1 °C per cycle) for 1 min and 72 °C for 4.5 min continued by 14 cycles of 95 °C for 15 s, 52 °C for 1 min, 72 °C for 4.5 min, and then a final elongation of 72 °C for 1 min. Again, the PCR product obtained was purified using the QIAquick PCR Purification kit (Qiagen), according to the manufacturer's instructions. The purified PCR product (approx. 5000 bp) and the vector pET20b(+) (Novagen) were digested using the restriction enzymes *NdeI* and *XhoI*. T4-DNA-ligase was used for the ligation of the PCR product digested and the vector, and resulted in the plasmid pET20b(+)_{*pepN_L1_pepX*}. This vector was transformed into competent *E. coli* XL1 Blue cells via heat shock and plated onto LB_{amp}-agar plates (tryptone 10 g L^{−1}, yeast extract 5 g L^{−1}, NaCl 5 g L^{−1}, ampicillin 100 µg mL^{−1}). After cultivation overnight at 37 °C, single colonies were picked and cultivated in 5 mL LB_{amp} medium overnight at 37 °C. Afterwards, the plasmids were isolated using the GeneJET Plasmid Miniprep kit (Fermentas), according to the manufacturer's instructions. The plasmids obtained were used for sequencing (SRD—Scientific Research and Development GmbH; Bad Homburg, Germany).

In the following, the fusion protein PepN-L1-PepX will always be called FUS-PepN-PepX. The terms “FUS-PepN activity” and “FUS-PepX activity” describe the particular activity in the fusion protein determined either with the substrate H-Ala-*p*NA (specific for PepN activity) or with the substrate H-Ala-Pro-*p*NA (specific for PepX activity), respectively.

Expression of the recombinant fusion protein FUS-PepN-PepX in *E. coli* BL21(DE3)

Transformed *E. coli* BL21(DE3) was grown in 2xYT medium (tryptone 16 g L^{−1}, yeast extract 10 g L^{−1}, NaCl 5 g L^{−1}) 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 preculture (5 mL) was grown overnight and the second (35 mL) and third (350 mL) were each cultivated for 15 h. The main culture (3.5 L) was grown in a tabletop bioreactor system (Minifors) and inoculated with 10 % (v/v) of the preculture. The pH value of the bioreactor cultivation was kept at pH 7.0 using 2 M NaOH and 2 M

H₃PO₄. The O₂ concentration (pO₂) dissolved in the medium was maintained above 30 % saturation by the regulation of the stirrer speed (400–1050 rpm). The aeration rate was 1 vvm. Samples were taken during the cultivation to analyze the optical density (OD_{600 nm}), the bio dry mass (BDM), and the glucose concentration, as described previously (Stressler et al. 2012). When the OD_{600 nm} value reached 5, the temperature was maintained at 30 °C to minimize the formation of inclusion bodies, and recombinant protein expression was induced by the addition of 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). The culture was harvested after 9.5 h of cultivation, as described previously (Stressler et al. 2012) and stored at –20 °C.

Samples (10 mL) were taken at various time points during cultivation to determine the FUS-PepN and FUS-PepX activity (see below) in the cell-free extract individually. Consequently, the samples were centrifuged (8000×g, 10 min, 4 °C) and the cell pellets were suspended in a 0.9 % (w/v) NaCl solution (5 mL). After centrifugation (see above), the cell pellets were suspended in Na/K-phosphate buffer (50 mM; pH 6.5). Subsequently, the cell disruption was realized by sonification (UP200S ultrasonic processor, Dr. Hielscher, Berlin, Germany; 20 cycles containing 1 min disruption, 1 min break) on ice.

Purification of FUS-PepN-PepX

Due to the attached His₆-tag, the fusion protein FUS-PepN-PepX was purified by Ni²⁺-affinity chromatography using an ÄKTA-FPLC system (GE Healthcare). At first, a cell suspension (15 % (w/v)) in binding buffer (BB: 50 mM Tris/HCl + 500 mM NaCl + 40 mM imidazole, pH 6.5) was prepared for cell disruption (see above). The supernatant (cell-free extract) after centrifugation (8000×g, 20 min, 4 °C) and filtration (0.45 μ m) was applied to the Ni²⁺ affinity column (IDA_{low}, KNAUER Wissenschaftliche Geräte GmbH, Berlin, Germany; 1 column volume (CV)=18.5 mL). An amount of 20 mL of the cell-free extract was injected at a flow rate of 1 mL min^{–1} using buffer BB. Afterwards, unbound protein was washed out for 9 CV at a flow rate of 2 mL min^{–1}. Bound protein was eluted with elution buffer (EB: 50 mM Tris/HCl + 500 mM NaCl + 500 mM imidazole, pH 6.5) by a linear gradient (4 CV) to 100 % EB at a flow rate of 2 mL min^{–1} and detected at 280 nm. Fractions (5 mL) were collected and tested for FUS-PepX activity. Active fractions were pooled, desalted into Na/K-phosphate buffer (50 mM; pH 6.5) using PD-10 columns (GE Healthcare), and stored at –20 °C until use.

Polyacrylamide gel electrophoresis

The samples were divided into soluble (supernatant) and insoluble (pellet) fractions after cell disruption. These fractions

and purified FUS-PepN-PepX (also soluble) were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (6 % separating gel) (Laemmli 1970). An amount of 5 μ g protein (Bradford 1976) or 7.5 μ g protein (Smith et al. 1985) was applied to gel in the case of the soluble or insoluble fractions, respectively. Bovine serum albumin was used as a standard for both protein determination methods (Bradford 1976; Smith et al. 1985). A commercial molecular mass protein mixture was used as a reference for molecular mass estimation (Spectra™ Multicolor High Range Protein Ladder; Thermo Scientific). Gels were stained with Coomassie Brilliant Blue for protein detection.

Native PAGE was conducted on ice using a commercial gel (4–15 % Mini-PROTEAN® TGX; Bio-Rad) with samples after purification (5 μ g protein; Bradford 1976). A native standard molecular mass protein mixture was obtained from SERVA Electrophoresis. The particular lanes of the gel were stained with Coomassie Brilliant Blue for protein detection. Additionally, some lanes were activity stained to verify the FUS-PepN and FUS-PepX activity. Therefore, a method described previously (Stressler et al. 2013b) was used with minor modifications: The substrates H-Ala-pNA (7.5 mg) and H-Ala-Pro-pNA (5 mg) were individually dissolved in dimethyl sulfoxide (DMSO; 250 μ L) and diluted to a final volume of 10 mL using Na/K-phosphate buffer (50 mM; pH 6.5). The incubation of the particular lanes of the native PAGE was realized at 37 °C until yellow bands were observed. The subsequent recoloring to violet-colored bands was performed as described previously (Božić and Vujčić 2005; Stressler et al. 2013b).

Isoelectric focusing

Isoelectric focusing of purified FUS-PepN-PepX was realized using the PhastSystem™ (GE Healthcare). A commercial gel (PhastGel IEF 3-9; GE Healthcare) was used. The preparation and electrophoretic separation was realized according to the manufacturer's instructions (Pharmacia LKB Biotechnology, Separation File No. 100 PhastSystem™, IEF and electrophoretic titration curve analysis). The sample (1 μ g_{Protein} μ L^{–1}) and the reference (see below) were applied to the gel using the PhastGel sample applicator (1 μ L; GE Healthcare). A commercial protein mixture (Broad pI Kit, pH 3–10; GE Healthcare) was used as a reference for isoelectric point (IEP) determination. Gels were stained with Coomassie Brilliant Blue for protein detection.

Size exclusion chromatography (SEC)

The native molecular mass determination of the purified fusion protein by size exclusion chromatography (SEC) was realized using a SpectraSYSTEMS™ HPLC system (Thermo Scientific, Dreieich, Germany) equipped with a

SEC-Yarra 4000 column (300 × 7.8 mm ID; Phenomenex, Aschaffenburg, Germany). The injection volume was 5 µL (1 mg_{protein} mL⁻¹) and the flow rate was 1 mL min⁻¹ using Na/K-phosphate buffer (50 mM, pH 6.8) containing NaCl (300 mM) as an eluent. Eluted protein was detected at 280 nm. Standard proteins (Gel Filtration HMW and LMW Calibration Kit, GE Healthcare) were used as references for molecular mass determination.

Standard enzyme activity assay

FUS-PepN or FUS-PepX activity was individually determined in the standard assay using H-Ala-*p*NA or H-Ala-Pro-*p*NA as a specific substrate, respectively. The standard assay was performed as described previously (Stressler et al. 2013b) with minor modifications: Initially, 50.5 µL enzyme solution was added to 177 µL Na/K-phosphate buffer (50 mM; pH 6.5). After incubation for 10 min at 37 °C, 12.5 µL of the particular substrate solution (FUS-PepN activity 7.5 mg_{H-Ala-*p*NA} mL_{DMSO}⁻¹; FUS-PepX activity 5 mg_{H-Ala-Pro-*p*NA} mL_{DMSO}⁻¹) was added to the reaction mixture. The reaction was terminated by adding 50 µL acetic acid (50 % (v/v)) to the sample. After centrifugation (8000×g, 5 min, 4 °C), 240 µL of the supernatant was transferred into a microtiter plate and the absorption was measured (Multiskan FC, Thermo Scientific, Braunschweig, Germany) at 405 nm. One katal (kat) of FUS-PepN or FUS-PepX activity was defined as the release of 1 mol *p*-nitroanilin s⁻¹. The specific activity of a particular sample was determined by dividing the volumetric activity by the corresponding protein content (Bradford 1976). Thus, the specific activity during the bioreactor cultivation is referred to the protein content in the supernatant after cell disruption. All other specific activity values are related to the samples after purification.

Biochemical characterization of FUS-PepN-PepX

The purified FUS-PepN-PepX was characterized. The standard assay was used unless otherwise stated.

Influence of pH and temperature on the initial activity of FUS-PepN-PepX and temperature stability

The pH was varied in the range between 5.0 and 9.0. All buffers had a concentration of 50 mM, and the following buffers were used: Na/K-phosphate (pH 5.0–7.0), bis-Tris-propane/HCl (pH 6.5–7.5) and Tris/HCl (pH 7.5–9.0). In contrast to the standard assay, the temperature was varied between 10 and 70 °C for optimum temperature determination. The enzyme preparation was incubated at 0, 37, and 50 °C for up to 2 weeks, and samples were taken several times for the temperature stability. Sodium azide (0.1 % (w/v)) was added to prevent microbial growth.

Influence of NaCl on FUS-PepN-PepX

In contrast to the standard assay, the buffer (50 mM Na/K-phosphate, pH 6.5) contained different NaCl concentrations (0–2.5 M in the final assay).

Influence of organic solvents, metal salts and other reagents on FUS-PepN-PepX

The assay conditions were identical to the standard assay, except that 24 µL of the test substance and 153 µL buffer were used together, instead of 177 µL buffer. The concentration of the solvents was 10 % (v/v) in the final assay. The concentration of metal salts and other reagents in the final assay varied between 0.0001 and 10 mM. They were dissolved in H₂O_{dd}.

Determination of the kinetic parameters of FUS-PepN-PepX

The kinetic parameters of FUS-PepN-PepX were individually determined using H-Ala-*p*NA and H-Ala-Pro-*p*NA as substrates. Standard activity assay conditions were used in which the final substrate concentration ranged from 0.01 to 6.2 mM depending on the particular enzyme activity and substrate. The results were plotted according to Michaelis-Menten, and the kinetic parameters were calculated either as described previously (Stressler et al. 2013b), by Hanes linearization or by nonlinear regression fitting using SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA).

Hydrolysis of casein with Alcalase® and the fusion protein FUS-PepN-PepX

The prehydrolysis of casein was performed using Alcalase® 2.4 L (Novozymes, Bagsvaerd, Denmark). In the second step, the purified fusion protein was added.

Determination of the initial activity of Alcalase® with o-phthaldialdehyde (OPA)

The initial enzymatic activity of Alcalase® 2.4 L was determined as described previously (Stressler et al. 2013b), with minor modifications using casein (1 % (w/v)) in Na/K-phosphate buffer (50 mM; pH 7.0) as a substrate. The substrate solution (250 µL) was pre-incubated for 10 min at 37 °C and, subsequently, 25 µL of diluted Alcalase® 2.4 L was added. The following reactions were realized at 37 °C and terminated at various time points (0.5–20 min) with the addition of 25 µL trichloroacetic acid (TCA; 2 M). After centrifugation (8000×g, 5 min), 25 µL of the supernatant was transferred to a microtiter plate, and 175 µL of the OPA reagent (Nielsen et al., 2001) was added. After exactly 1 min, the absorption was measured (Multiskan FC, Thermo Scientific,

Braunschweig, Germany) at 340 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 releasing 1 mol of L-serine equivalent amino groups per second.

Prehydrolysis of casein with Alcalase®

A prehydrolysis of casein using Alcalase® 2.4 L was performed as described previously (Stressler et al. 2013b; Stressler et al. 2015) with minor modifications. Therefore, casein (5 g) was suspended in 200 mL Na/K-phosphate buffer (50 mM; pH 6.5). Alcalase® (440 nkat mL_{Casein solution}⁻¹) was added after pre-incubation of the casein solution (37 °C, 30 min). Samples (450 µL) were taken at various time points during hydrolysis until no increase in serine equivalents was observed. The samples were added to 50 µL SDS (10 % (w/v)) and heated to 80 °C for 10 min for enzyme inactivation. After centrifugation (8000×g, 5 min), the samples were analyzed using the OPA assay described above. In addition, samples (1 mL) were taken directly inactivated at 80 °C without the addition of SDS. These samples were used for the determination of the antioxidative effect. All inactivated samples were stored at -20 °C for analysis conducted later. At the end, the hydrolyzed casein solution was heated to 85 °C for 30 min and stored at -20 °C.

Application of FUS-PepN-PepX on a prehydrolyzed casein solution

The final prehydrolyzed casein solution (see above) was used as a substrate for further hydrolysis with FUS-PepN-PepX. Sodium azide (0.1 % (w/v)) was added to the prehydrolyzed casein solution (40 mL) to prevent microbial growth, and it was pre-incubated at 37 °C for 30 min. Subsequently, the fusion protein was added with a standardized FUS-PepN activity of 5 nkat_{H-Ala-pNA} mL_{Casein solution}⁻¹ (corresponds to a FUS-PepX activity of 11 nkat_{H-Ala-Pro-pNA} mL_{Casein solution}⁻¹). Samples were taken over a time period of 24 h and treated as described above. At the end, the hydrolyzed casein solution was heated to 85 °C for 30 min and stored at -20 °C.

Reversed phase HPLC analysis of the casein hydrolysates

The analysis of the casein hydrolysates (determination of released amino acids) was realized using a PLATINblue UHPLC system (KNAUER Wissenschaftliche Geräte GmbH) equipped with a RP-C8 column (BlueOrchid C8, 1.8 µm, 100×2 mm ID, KNAUER Wissenschaftliche Geräte GmbH), as described previously (Merz et al. 2015a). Before the automated OPA derivatization was realized, the

samples (without SDS) from the casein hydrolysis were diluted (1:20) and the internal standard (ISTD; ethanolamine, final concentration 100 µM) was added. The following OPA derivatization was automated by the PLATINblue autosampler AS-1. The temperature in the autosampler was set to 22 °C for the derivatization. A volume of 75 µL OPA reagent was added to the sample prepared and mixed thoroughly. The OPA reagent consisted of 25 mM *ortho*-phthalaldehyde, 43 mM β-mercaptoethanol, and 30 % (v/v) methanol in 85 mM sodium tetraborate buffer (pH 9.6, adjusted with NaOH). The derivatization time was 1 min and, afterwards, 50 µL of neutralization buffer (1 M sodium acetate pH 5.0) was added and, again, the mixture was mixed thoroughly. A sample volume of 5 µL was injected into the column for chromatography. Solvent A consisted of 70 mM disodium hydrogen phosphate (adjusted with HCl to pH 6.5) and 5 % (v/v) acetonitrile. Solvent B consisted of H₂O_{dd}/methanol/acetonitrile [40:30:30 (v/v/v)]. The conditions of gradient elution were as follows: 0–4 min (A, 100 %; B, 0 %), 4–13.5 min (A, 0 %; B, 100 %), 13.5–14 min (A, 0 %; B, 100 %), 14–14.1 min (A, 100 %; B, 0 %), and 14.1–15 min (A, 0 %; B, 100 %). The flow rate was 0.7 mL min⁻¹ and derivatives were detected at 340 nm. The column temperature was set at 45 °C. The assignment of the peaks detected was realized over the retention time of reference amino acids.

Antioxidative effect of the casein hydrolysates

The antioxidative effect of the different samples during the hydrolysis was determined using the ABTS⁺ [2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt] decolorization assay, according to the literature (Re et al. 1999) with modifications (Eisele et al. 2013; Stressler et al. 2013a). The ABTS⁺ stock solution was prepared with ABTS (7 mM) and ammonium peroxodisulfate (APS; 2.45 mM) in H₂O_{dd}. The stock solution was allowed to react for 12–16 h in the dark and was, subsequently, used for 2 days. Before usage, the stock solution was diluted with phosphate-buffered saline (PBS; pH 7.4) to an absorption of 0.700±0.05 at 734 nm. This diluted ABTS⁺ solution (1000 µL) was mixed with the particular diluted samples from the casein hydrolysis (10 µL; 5 µg_{Casein} µL⁻¹) and incubated at 30 °C in the dark for 15 min. Afterwards, the samples were measured at 734 nm. Na/K-phosphate buffer instead of the sample was used as a reference.

The ABTS-IC₅₀ value was defined as the amount of hydrolysate required to reduce the absorbance of ABTS⁺ to 50 %. Therefore, the final sample after FUS-PepN-PepX treatment was diluted to final concentrations between 0.25 and 10 µg_{Casein} µL⁻¹ and used in the ABTS⁺ assay as described above.

Statistical analysis

The standard deviation was used for data evaluation and calculated with Excel (Microsoft, Redmond, USA). All experiments were conducted at least in duplicate, with three independent measurements. The standard deviation was always below 5 %.

Results

In this study, a fusion protein consisting of the exopeptidases PepN and PepX, originating from *Lb. helveticus* ATCC 12046, was recombinantly produced in *E. coli* and biochemically characterized after purification. In addition, the fusion protein was applied for casein hydrolysis and the antioxidative capacity of the hydrolysate obtained was investigated. The fusion protein PepN-L1-PepX will be abbreviated as FUS-PepN-PepX in the following.

Sequencing and heterologous expression of the fusion protein FUS-PepN-PepX

The FUS-PepN-PepX expression vector (pET20b(+)_*pepX_L1_pepN*) was constructed by linking the genes of *pepN* and *pepX* using a linker called L1 (Kadow et al. 2014). The translated linker DNA sequence results in ten amino acids SSGLVPRGSH. Due to the cloning strategy chosen, a C-terminal His₆-tag was attached. The fusion gene constructed was sequenced (see Supplemental Material Figure S1) and exhibited 100 % identity to theoretical sequences of the genes of *pepN* and *pepX* (Stressler et al. 2013b) as well as the linker.

The fusion protein FUS-PepN-PepX was expressed using the expression host *E. coli* BL21(DE3) in soluble and insoluble form (inclusion bodies) in a ratio of approximately 1:1. However, the soluble enzyme activity obtained (Fig. 1) was acceptable, and thus, no experiments were performed to increase the soluble amount of enzyme in this study. FUS-PepN-PepX showed two distinguished activities either with H-Ala-*p*NA or with H-Ala-Pro-*p*NA as specific substrates. During the bioreactor cultivation (Fig. 1), the glucose was completely consumed after about 7 h and, subsequently, the cells entered the stationary growth phase. The OD_{600 nm} value increased up to 32 during cultivation (corresponds to a cell dry weight of about 10 g L⁻¹), and the volumetric activity was 248 and 228 $\mu\text{kat L}_{\text{Culture}}^{-1}$, for separate FUS-PepN and FUS-PepX activity, respectively, by entering the stationary growth phase (Fig. 1a). During the latter, the volumetric activity decreased caused by degradation of the fusion protein, as seen in the SDS-PAGE analysis (data not shown). The highest specific activity for FUS-PepN ($74 \text{ nkat}_{\text{H-Ala-pNA}} \text{ mg}_{\text{Protein}}^{-1}$) and FUS-PepX ($83 \text{ nkat}_{\text{H-Ala-Pro-pNA}} \text{ mg}_{\text{Protein}}^{-1}$) was determined

after about 5.5 h of cultivation (Fig. 1b). Afterwards, the specific activity decrease most probably caused by a stronger expression of *E. coli*'s own proteins in comparison to the recombinant fusion protein.

Purification of FUS-PepN-PepX, molecular mass determination, and IEP

The His₆-tagged fusion protein FUS-PepN-PepX was purified using a FPLC procedure based on Ni²⁺ IMAC chromatography resin. After purification, a specific activity for FUS-PepN of $280 \text{ nkat}_{\text{H-Ala-pNA}} \text{ mg}_{\text{Protein}}^{-1}$ and for FUS-PepX of $600 \text{ nkat}_{\text{H-Ala-Pro-pNA}} \text{ mg}_{\text{Protein}}^{-1}$ was achieved, respectively. The purity and the molecular mass of the monomeric fusion protein were determined by SDS-PAGE (Fig. 2a). The fusion protein was concentrated by the purification (Fig. 2a, lane 1 and lane 3), and no FUS-PepN-PepX was detected in the flow-through (Fig. 2a, lane 2). The molecular mass of the monomeric FUS-PepN-PepX was determined at about 190 kDa (Fig. 2a, lane 3). This is in accordance with the theoretical molecular mass of 188.3 kDa (based on the amino acid sequence including the His₆-tag). In addition, a native PAGE with activity staining was performed (data not shown). An activity was detected for both substrates (H-Ala-*p*NA and H-Ala-Pro-*p*NA), indicating that both enzyme activities were present in the fusion protein. The molecular mass determined by native PAGE was about 300 kDa. In addition, SEC experiments were performed to analyze the purity and the native mass of the fusion protein (Fig. 2b). The chromatogram showed a defined single peak, indicating the high purity of purified fusion protein. The molecular mass was determined to be about 600 kDa. This is in disagreement with the molecular mass determined by native PAGE (about 300 kDa). However, the fact that these methods are based on different principles should be taken into account. In the case of the native PAGE, the charge and conformation of the protein plays an important role during the separation process, whereas with the SEC analysis, it is the diffusion into and out of the pores of the chromatography resin. The IEP of the fusion protein was determined experimentally to be 5.19. Based on the amino acid sequence, a theoretical IEP of 6.10 was calculated (ExPASy server; Compute pI/Mw tool). This difference can be explained by the fact that by theoretical consideration, all amino acids of the protein were considered and not only the surface-related amino acids of the protein, which are decisive for the experimental IEP.

Effect of pH and temperature on the initial FUS-PepN-PepX activity and temperature stability

The optimum pH of the FUS-PepN and FUS-PepX activity was determined using different buffers (all 50 mM; Fig. 3a, b). The highest FUS-PepN activity was detected using Na/K-

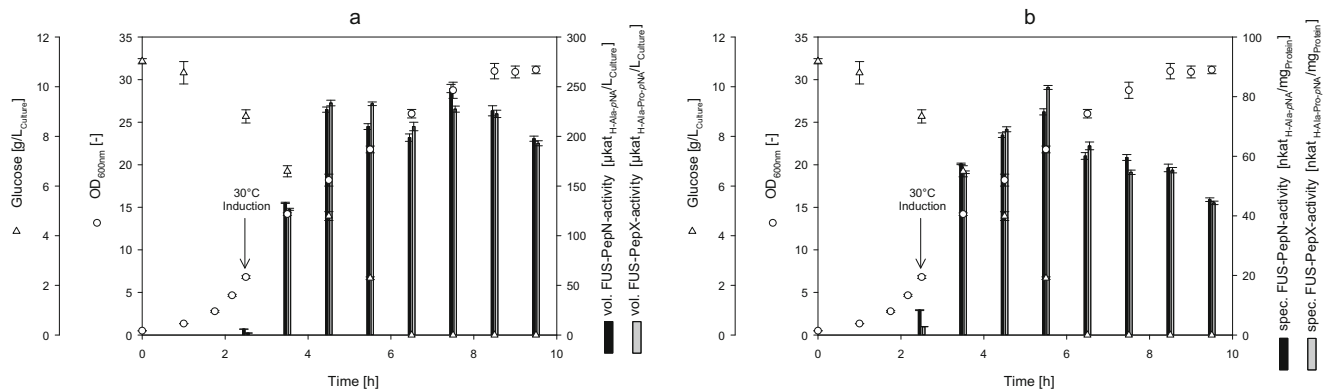


Fig. 1 Course of the bioreactor cultivation (working volume, 3.5 L) of recombinant *E. coli* BL21(DE3) for the production of the fusion protein FUS-PepN-PepX. Cultivations began at 37 °C and shifted to 30 °C with

simultaneous induction using IPTG (see arrow). The volumetric enzyme activity (a) and the specific enzyme activity (b) are shown. The means $\pm$ standard deviation of three independent measurements are presented

phosphate buffer (pH 6.5; 100 % = 450.6 nkat_{H-Ala-pNA} mL⁻¹; Fig. 3a). At the same pH value, but using bis-Tris-propane/HCl buffer, the activity was 82 %. The residual activity was 47 % at a pH value of 5.0, and there was almost no FUS-PepN activity (5 %) determined at a pH value of 9.0. A broad optimum pH range was observed for the FUS-PepX activity (Fig. 3b; 100 % = 918.4 nkat_{H-Ala-Pro-pNA} mL⁻¹). The relative activity using Na/K-phosphate buffer between pH 5.0 and 7.0 was 93 and 88 %, respectively. An activity of 98 % was determined at pH 6.5. The residual activity was 36 % at a more basic pH value of 9.0. The optimum temperature for FUS-PepN was determined at 35 °C (100 % = 369.5 nkat_{H-Ala-pNA} mL⁻¹), as seen in Fig. 3c. At a higher temperature

(40 °C), 88 % of the maximum activity was achieved. Almost no activity (1.8 %) was detected at 60 °C. The optimum for the FUS-PepX activity was determined at 50 °C (100 % = 1384 nkat_{H-Ala-Pro-pNA} mL⁻¹; Fig. 3d). A minor lower activity (94 %) was detected at 55 °C. A residual activity of 17 % was measured at 70 °C. The temperature stability of FUS-PepN-PepX was determined at 0 °C as well as at 37 and 50 °C (Fig. 3e, f). At 0 °C (on ice), both enzymes were quite stable over the analysis time (14 days) with a residual activity of 89 and 70 % for FUS-PepN and FUS-PepX activity, respectively. Interestingly, the FUS-PepN activity at 37 °C, near the optimum temperature of FUS-PepN, was higher

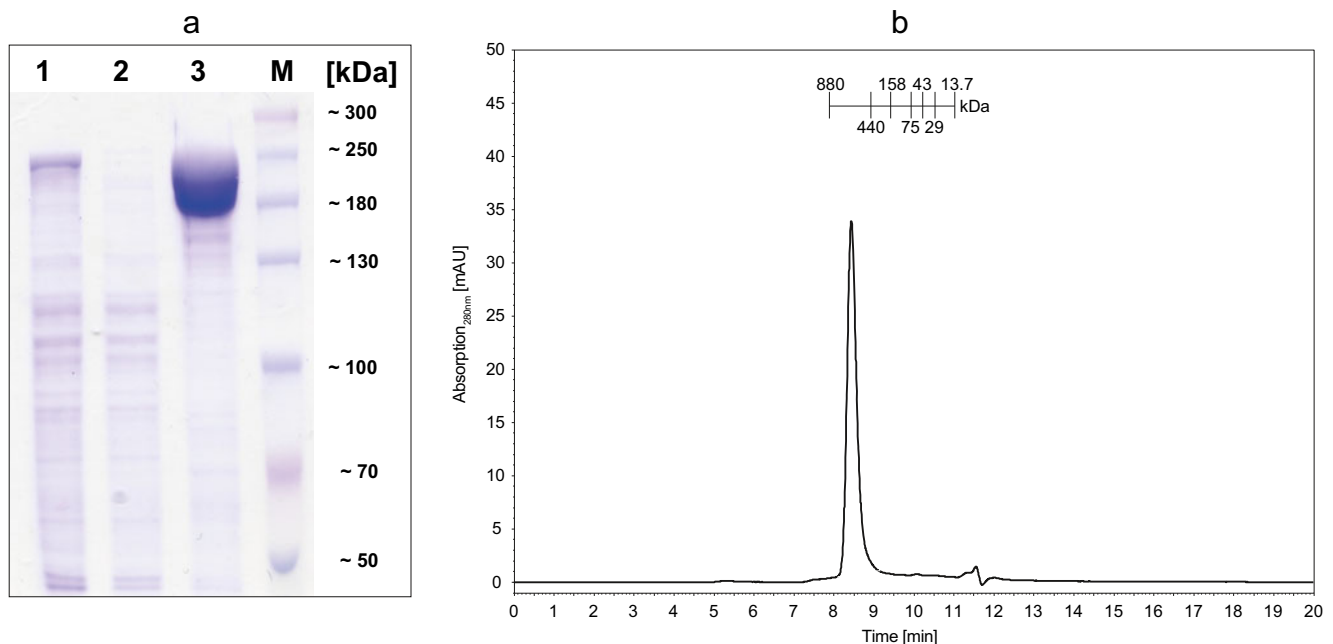


Fig. 2 SDS-PAGE of the fusion protein FUS-PepN-PepX purification (a) and HPLC-SEC (SEC-Yarra 4000 column, Phenomenex) analysis of the purified fusion protein (b). The cell extract after cell disruption (a; lane 1), the flow-through of the purification (a; lane 2), and the purified

protein (a; lane 3) are shown. The molecular mass marker is presented in lane M (a). The molecular mass scale (b) was prepared using standard proteins (Gel Filtration HMW and LMW Calibration Kit, GE Healthcare)

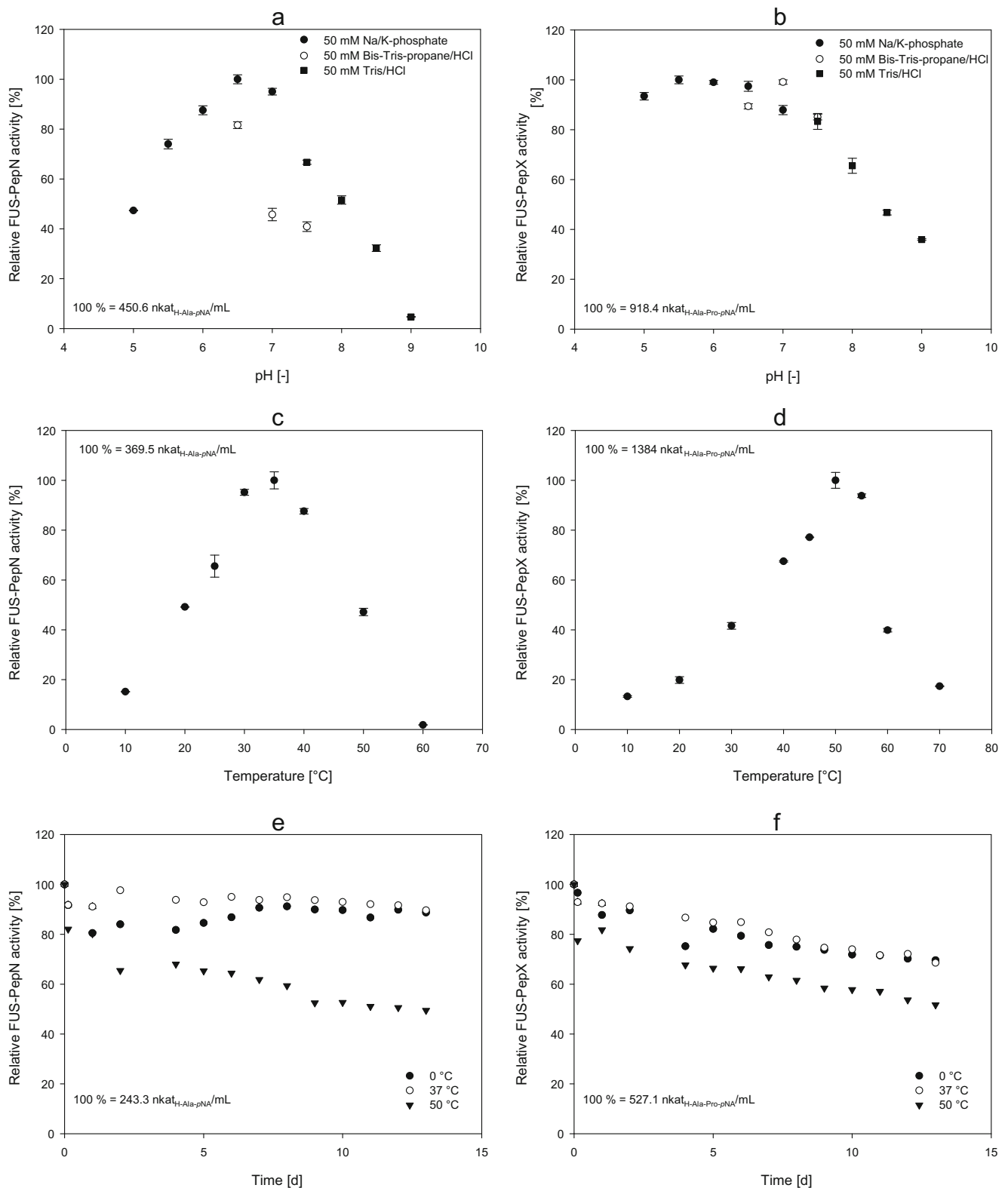


Fig. 3 Determination of optimum pH (a, b), optimum temperature (c, d), and temperature stability (e, f) of fusion protein FUS-PepN-PepX. FUS-PepN activity: a, c, e; FUS-PepX activity: b, d, f. The means $\pm$ standard deviation of three independent measurements are presented

(88 %) than the FUS-PepX activity (69 %). Both enzymes showed similar residual activity of about 50 % after 14 days at 50 °C, the optimum temperature of

FUS-PepX. These results demonstrated the high thermal stability of the fusion protein and its potential for application in protein hydrolysis processes.

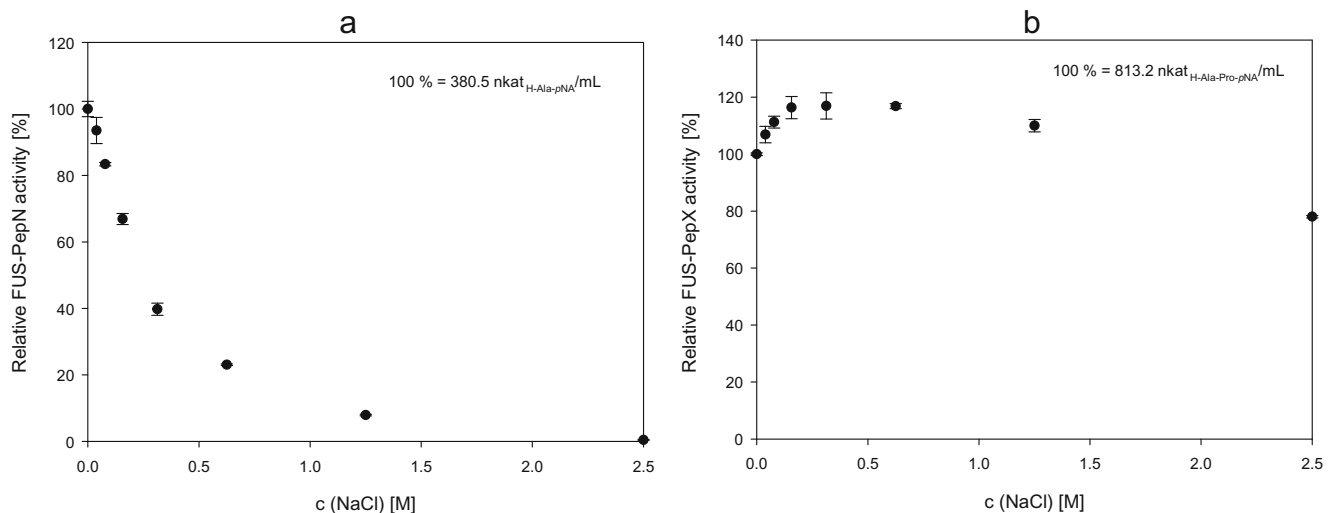


Fig. 4 Determination of the influence of NaCl on the FUS-PepN activity (a) and FUS-PepX activity (b) of fusion protein FUS-PepN-PepX. The means $\pm$ standard deviation of three independent measurements are presented

Influence of organic solvents, other reagents, and metal salts on the FUS-PepN-PepX activity

Due to the low solubility of the *p*NA substrates in water, they were dissolved in dimethyl sulfoxide (DMSO) and, thus, the *p*NA standard assay contained 5.2 % (*v/v*) DMSO. To determine the influence of each organic solvent on the activity of the fusion protein, the particular activity was measured in the presence of an additional 10 % (*v/v*) of the particular solvent (Table 1). The activity value after the addition of 10 % (*v/v*) H₂O_{dd} was used as a reference (100 %). The FUS-PepN activity was not reduced by additional DMSO, whereas the FUS-PepX activity was reduced to 63 %. However, all other solvents tested (ethanol, acetone, dimethylformamide (DMF)) had a stronger negative effect on the enzyme activity of FUS-PepN-PepX. Thus, DMSO is the most suitable organic solvent for substrates that are not soluble in water. In addition to the organic solvents, the influence of ethylenediaminetetraacetic acid (EDTA) β -mercaptoethanol on FUS-PepN-PepX was tested. Due to the fact that PepN is a metallopeptidase, EDTA reduced the activity remarkably. The FUS-PepX activity was not inhibited, because PepX is a serine peptidase (Stressler et al. 2013b). The disulfide bond-reducing reagent β -mercaptoethanol had no negative influence on either enzyme activity. Finally, different metal salts were tested for their influence on FUS-PepN-PepX. All metal salts were used as chlorides to prevent any influence of the anion. The FUS-PepX activity was less affected by the metal salts than the FUS-PepN activity. The addition of CaCl₂, for example, up to a concentration of 10 mM, had no remarkable influence on the FUS-PepX activity, but the FUS-PepN activity was reduced to 5 %. Both CoCl₂ and NiCl₂ had an effect on the activity of FUS-PepN-PepX, but again, FUS-PepN activity was more affected. Due to the fact that NaCl is sometimes

present in food protein hydrolysis processes to prevent microbial growth, the effect of NaCl on the FUS-PepN-PepX activity was investigated in more detail (Fig. 4). The FUS-PepN activity was decreased remarkably even at low concentrations (e.g., 0.3 mM; 39 % residual activity), as seen in Fig. 4a. At 2.5 M NaCl, almost no FUS-PepN activity (0.5 %) was determined. The picture changed for the FUS-PepX activity in the presence of NaCl. Up to a concentration of 0.3 mM, the FUS-PepX activity increased to 117 % and decreased with higher concentrations. At 2.5 M NaCl, the FUS-PepX activity was 78 % (Fig. 4b).

Determination of kinetic parameters of FUS-PepN-PepX

The kinetic parameters of FUS-PepN-PepX ($V_{\max}$, K_M , and K_{IS}) were determined using H-Ala-*p*NA and H-Ala-Pro-*p*NA as specific substrates. The particular specific activities were plotted according to Michaelis-Menten (Fig. 5) and the kinetic parameters were calculated (Table 2). A strong substrate inhibition was determined for FUS-PepN activity using the substrate H-Ala-*p*NA, as seen in Fig. 5a. No substrate inhibition was determined for the FUS-PepX activity with H-Ala-Pro-*p*NA as a substrate (Fig. 5b). No remarkable differences in the kinetic parameters were observed by using Michaelis-Menten plot/Hanes linearization and nonlinear regression fitting using SigmaPlot.

Hydrolysis of casein with Alcalase® and the fusion protein FUS-PepN-PepX

At first, casein (2.5 % (*w/v*)) was hydrolyzed with the commercial endopeptidase preparation Alcalase® 2.4 L, until no increase in the degree of hydrolysis was observed (Fig. 6a). This amount of serine equivalent (degree of hydrolysis) was

Table 1 Effect of various solvents, metal salts, and other reagents on the activity of the fusion protein FUS-PepN-PepX

	Substance	Concentration	Relative activity ^a [%]	
			FUS-PepN	FUS-PepX
Solvent [% (v/v)]	Acetone	10	34	35
	Ethanol	10	28	64
	DMSO	10	108	63
	DMF	10	43	20
Metal salt (mM)	CaCl ₂	0.1	102	97
		1.0	60	99
		10	5	93
	CoCl ₂	0.1	174	102
		1.0	103	107
		10	15	35
	ZnCl ₂	0.1	24	104
		1.0	15	94
		10	1	5
Reagent (mM)	β -Mercaptoethanol	0.1	105	104
		1.0	98	100
		10	104	104
	EDTA	0.0001	97	92
		0.001	84	93
		0.01	6	97

^a The value of 100 % (FUS-PepN, 336 nkat_{H-Ala-pNA} mL⁻¹; FUS-PepX: 812 nkat_{H-Ala-Pro-pNA} mL⁻¹) was determined in the presence of the solvent H₂O_{dd} without the additional substance; presented are the means of three independent measurements and the standard deviation was <5 %

set as 100 % relative degree of hydrolysis. The prehydrolysis with Alcalase[®] was necessary because PepN and PepX are exopeptidases, and thus, they cannot act on intact proteins. Before application of FUS-PepN-PepX, Alcalase[®] was inactivated by heat treatment. Directly after the application of the fusion protein, the relative degree of hydrolysis increased due to the action of the exopeptidases. After 24 h of

hydrolysis using FUS-PepN-PepX, the relative degree of hydrolysis was 230 % (Fig. 6a). This means that the relative degree of hydrolysis had increased by 130 % due to the action of FUS-PepN-PepX. The release of the amino acids was analyzed by HPLC (Fig. 6b). Beside others, the figure shows the casein substrate solution before prehydrolysis. No released amino acids were detectable. The peaks between 0 and

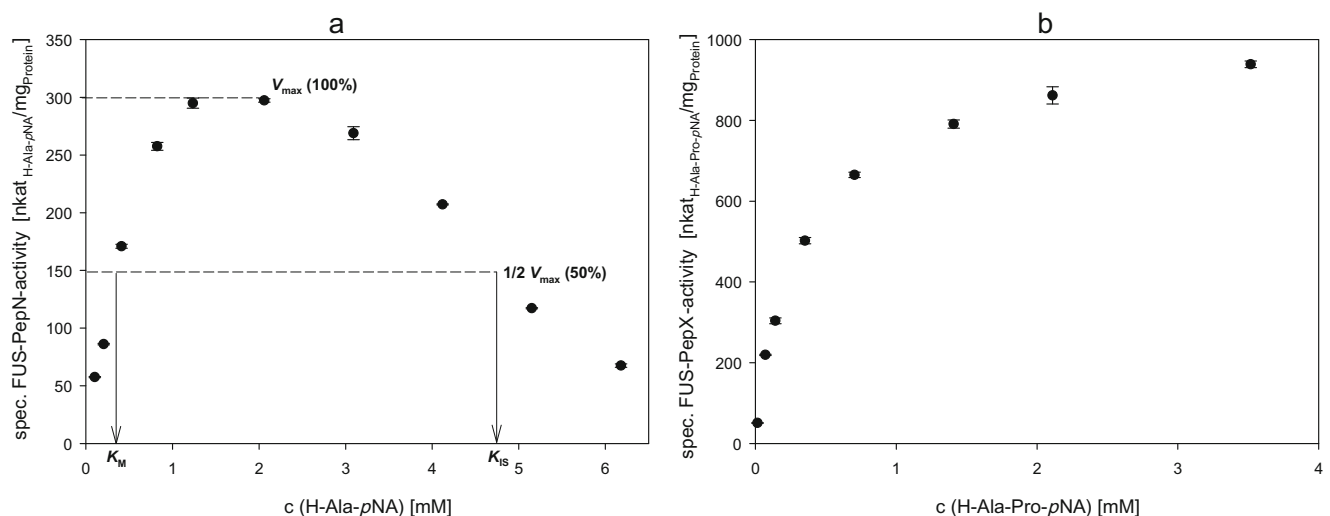


Fig. 5 Determination of the kinetic parameters of FUS-PepN (a) and FUS-PepX (b) from the fusion protein FUS-PepN-PepX. The Michaelis-Menten plots are displayed and the results presented are the means $\pm$ standard deviation of three independent measurements

Table 2 Kinetic parameters of the fusion protein FUS-PepN-PepX

	FUS-PepN ^a		FUS-PepX ^b	
	Michaelis-Menten	Nonlinear regression ^c	Hanes linearization	Nonlinear regression ^d
$V_{\max}$ (nkat mg _{Protein} ⁻¹)	297	314	1000	998
K_M (mM)	0.34	0.37	0.27	0.33
K_{IS} (mM)	4.74	4.65	n.a.	n.a.

The calculation of the kinetic parameters was performed according to Michaelis-Menten, Hanes linearization, and nonlinear regression fitting using SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA). Presented are the means of three independent measurements and the standard deviation was <5 %

n.a. not applicable

^a Substrate: H-Ala-pNA

^b Substrate: H-Ala-Pro-pNA

^c SigmaPlot: Weibull function (Murado and Prieto 2013)

^d SigmaPlot: Enzyme Kinetics Tool

2 min as well as 11.8 min belong to the derivatization reagents. The peak at 15.2 min is the added ISTD. Many peaks were detected after prehydrolysis with Alcalase®. Some of these peaks belong to released amino acids, but mainly to short peptides. A remarkable increase in the peak heights was observed after the application of the fusion protein FUS-PepN-PepX. The remarkable increase of the relative degree of hydrolysis and the peak heights result from the release of amino acids and X-Pro dipeptides from the prehydrolyzed casein caused by FUS-PepN-PepX. However, it is worth mentioning that the labeled peaks correspond to the retention times of the standard amino acids analyzed. Thus, it was not excludable that short peptides with a similar retention time were also present underneath the peaks. The fact that short peptides were present in the hydrolysate was visible due to the small peaks

between the labeled peaks. Thus, we decided not to quantify the amino acids released.

Antioxidative effect of the casein hydrolysates

The antioxidative effect of different stages of the casein hydrolysis was investigated using the ABTS^{•+} decolorization assay. All samples tested were dissolved to a casein content of 5 µg mL⁻¹ due to the high antioxidative effect of the final hydrolysate. A non-hydrolyzed casein solution showed an antioxidative effect of approximately 15 %, as seen in Fig. 7a. After the prehydrolysis using Alcalase®, the antioxidative effect increased to approximately 27 %. The application of the fusion protein FUS-PepN-PepX increased the antioxidative effect again to over 40 % at the end of the hydrolysis process.

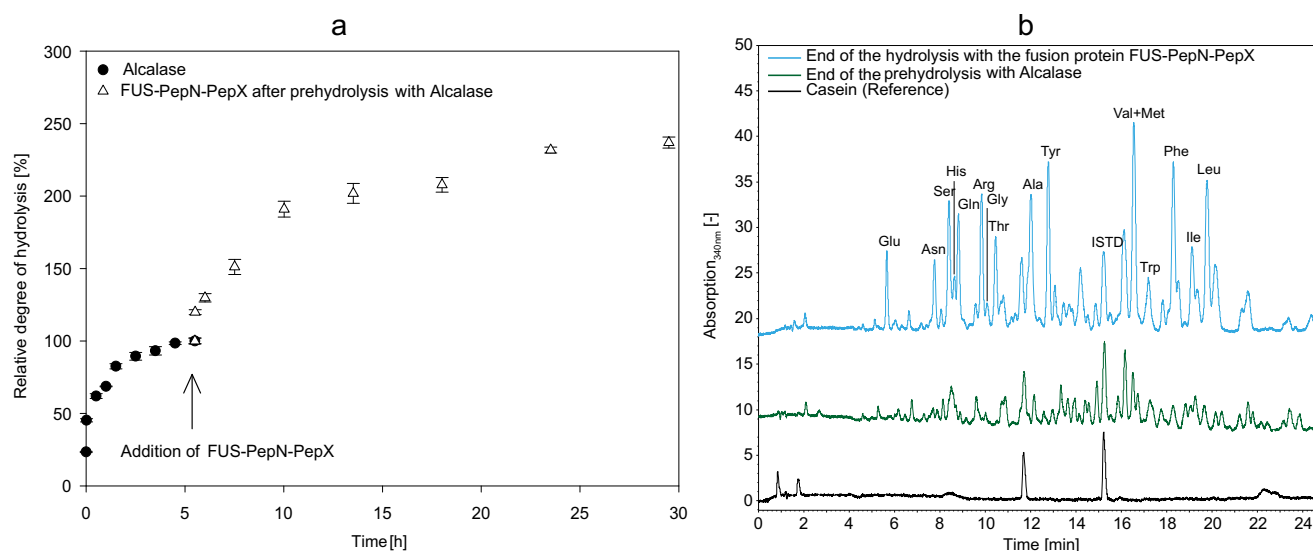


Fig. 6 Course of the increase of the relative degree of hydrolysis of a casein solution prehydrolyzed by Alcalase® and further hydrolyzed by the fusion protein FUS-PepN-PepX (a) and RP-HPLC chromatograms of the amino acids and short peptides released at different stages of hydrolysis (b)

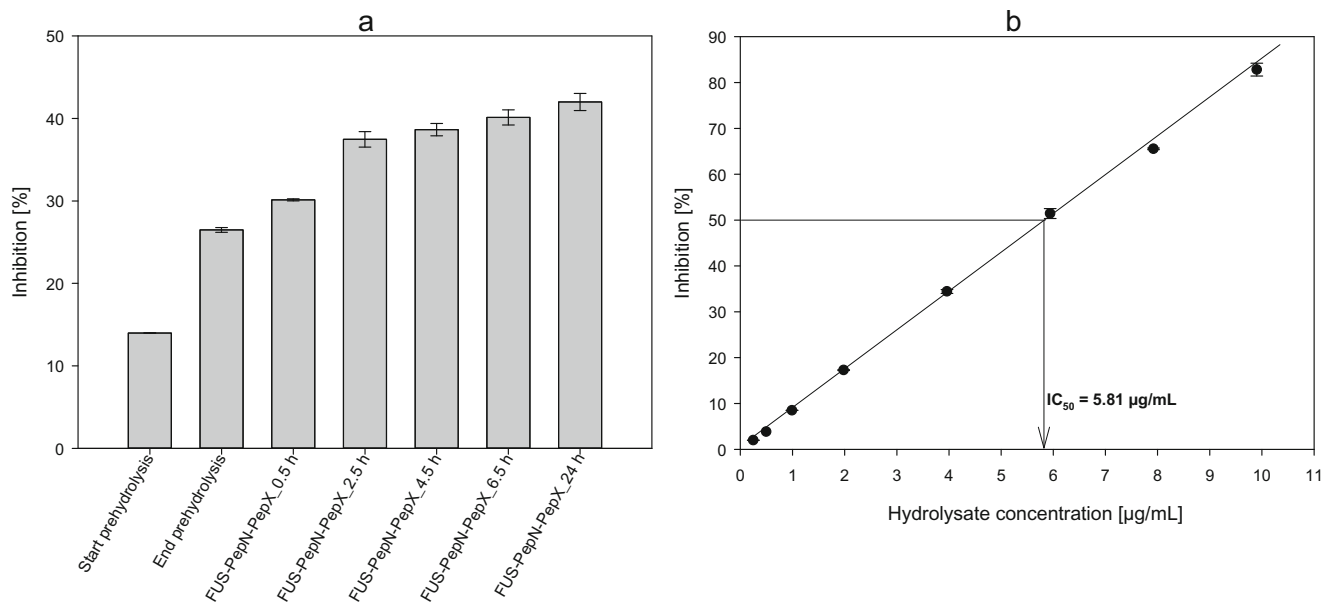


Fig. 7 Course of the antioxidative effect during different stages of casein hydrolysis with Alcalase® and the fusion protein FUS-PepN-PepX (a), and relationship between the hydrolysate concentration of the final hydrolysate and the antioxidative effect (b). In (a), the

casein(hydrolysate) concentration was standardized to $5 \mu\text{g mL}^{-1}$, and in (b), the hydrolysate concentration varied between 0.25 and $10 \mu\text{g mL}^{-1}$. The ABTS⁺ decolorization assay was used for both

However, the ABTS- IC_{50} value is more descriptive. Therefore, the final sample after FUS-PepN-PepX application was used and diluted to final casein(hydrolysate) concentrations between 0.25 and $10 \mu\text{g mL}^{-1}$. The ABTS- IC_{50} value of the hydrolyzed casein solution was low with $5.81 \mu\text{g mL}^{-1}$, as seen in Fig. 7b.

Discussion

In this work, a *pepN-pepX* fusion gene from *Lb. helveticus* ATCC 12046 was constructed by overlap extension PCR, cloned into the expression vector pET20b(+), and expressed using the expression host *E. coli* BL21(DE3). A linker, L1, linked both genes. The fusion protein PepN-L1-PepX produced, called FUS-PepN-PepX in the following, showed activity for both enzymes. Altogether, this study reported the first fusion protein of two exopeptidases and its application in casein hydrolysis.

Construction of fusion proteins

Fusing the target proteins with proteins, such as maltose-binding protein or affinity tags, e.g., His-tag, Arg-tag, or FLAG-tag, is a common method to facilitate the purification of the target protein (Terpe 2003). In addition, a target protein can be fused, for example, with green fluorescent protein (GFP) to specifically detect this molecule (Yuste 2005). Furthermore, different fusion proteins are applied as biopharmaceuticals (Schmidt 2009). In addition to these

applications, fusion proteins can consist of two enzymes and the protein produced should have both enzyme activities (Hong et al. 2006; Lu and Feng 2008; Trujillo et al. 1997). An important point is the usage of a so-called linker between both enzymes, because the direct fusion of both enzymes can lead to many undesirable results (Chen et al. 2013), such as misfolding of the fusion proteins (Zhao et al. 2008), low yields (Amet et al. 2009), or reduced activity (Bai et al. 2005; Bai and Shen 2006). In the current study, the linker L1 was used, which consists of ten amino acids (SSGLVPRGSH). The same linker was used in a previous study in which a monooxygenase was fused with a flavin reductase (Kadow et al. 2014). In preliminary experiments, our group used another linker (L2), which consisted of a five-times repetitive sequence of the amino acids GS. This linker was used following a previous study in which a six-times repetitive sequence of GS was used as a linker for constructing a fusion protein consisting of D-amino acid oxidase (DAAO) and glutaryl-7-aminocephalosporanic (GLA) (Luo et al. 2004). Due to the higher enzyme activity obtained using the linker L1, this linker was used for all further experiments. Beside the linker used, the orientation of fusion protein can also affect the catalytic activity of the fusion protein. This was shown for the fusion protein pair DAAO-linker-GLA (DLG) and GLA-linker-DAAO (GLD). The fusion protein DLA was not correctly folded and only DAAO activity was detected. In the case of ALD, both activities (DAAO and GLA) were determined (Luo et al. 2004). In further preliminary experiments, the orientation of the fusion protein was also changed and PepX-L1-PepN was created (Bsoul 2015). Again, activity was

detectable for both enzymes; however, the activity of the fusion protein PepN-L1-PepX was slightly higher. Thus, the fusion protein PepN-L1-PepX was used for all further experiments, because a direct comparison of the biochemical characteristics between both fusion proteins was not the focus of this study.

Comparison of the biochemical characteristics between the single enzymes and the fusion protein and application of the fusion protein in food protein hydrolysis

A great advantage of fusion proteins is the combination of different enzyme activities in one single molecule. However, it is not clear if the fusion protein has the desired activity and how the biochemical characteristics will change compared to the single enzymes. In the current study, the activity of both enzymes was present in the fusion protein FUS-PepN-PepX. During the characterization of the fusion protein, it was found that some biochemical characteristics changed compared to the single enzymes, but always in a positive way regarding application. Some selected characteristics of the fusion protein are compared to the single enzymes in Table 3 (Stressler et al. 2013b). Regarding the optimum pH values, the same values were determined for PepN activity (pH 6.5). By contrast, the

FUS-PepX activity showed a broader optimum (pH 5.5–6.5), compared to the single enzyme (pH 6.5). The improved pH stability within the range of pH 4.5–7.5 for FUS-PepX activity is remarkable. The residual activity was approximately 90 % at a pH value of 5.0, whereas a residual activity of about 45 % was detected for the single enzyme at the same pH (Stressler et al. 2013b). This is a great advantage, because food generally has an acid or neutral pH value. The optimum temperature was 35 °C for the FUS-PepN activity and 30 °C in the single enzyme. For PepX activity, the same optimum temperature of 50 °C was determined for FUS-PepX and the single enzyme (Stressler et al. 2013b). A more interesting characteristic for application than the optimum temperature of an enzyme is the temperature stability, especially for continuous processes. A sufficient stability at higher temperatures is desirable regarding the microbial stability of a continuous process. At 50 °C, the residual FUS-PepX activity was 50 % after 14 days of incubation. This was higher than for the single enzyme with about 40 % residual activity (Stressler et al. 2013b). In the case of PepN, no difference was observed between the single enzyme and FUS-PepN. Both showed a residual activity of about 50 % at 50 °C after 14 days. The microbial stability is generally an important point for later continuous processes. In addition to the temperature, the addition of NaCl also results in a higher microbial stability and is common in the food

Table 3 Comparison of selected characteristics of the single enzymes PepN and PepX and the fusion protein FUS-PepN-PepX

	Single enzymes ^a		Fusion protein ^b	
	PepN ^c	PepX ^d	FUS-PepN ^c	FUS-PepX ^d
Optimum pH [-]	6.5	6.5	6.5	5.5–6.5
Optimum temperature (°C)	30	50	35	50
Temperature stability [residual activity (%) at 50 °C after 14 days]	50	40	50	50
Influence NaCl [residual activity (%) at 2.5 M]	0	75	0	75
Influence ethanol [residual activity (%) at 10 % (v/v)]	18	78	28	64
Influence EDTA [residual activity (%) at 0.001 mM]	8	n.d.	84	93
Influence β -mercaptoethanol [residual activity (%) at 10 mM]	39	105	104	104
K_M (mM)	0.43	1.3	0.34	0.27
K_{IS} (mM)	5.72	n.a.	4.74	n.a.
Increase of relative degree of hydrolysis (%)				
Single enzyme application	100	12	n.a.	n.a.
Combined application		132		130

n.d. not determined, n.a. not applicable

^a Stressler et al. 2013b

^b Current study

^c Substrate: H-Ala-pNA

^d Substrate: H-Ala-Pro-pNA

industry. The addition of NaCl (150 g L^{-1} ; 2.57 M) to a wheat gluten suspension (37°C), for example, increased the time of microbial stability to 24 h, compared to 12 h without any addition (Merz et al. 2015b). However, the application of NaCl in combination with FUS-PepN-PepX is not recommended, because the FUS-PepN activity was strongly inhibited by NaCl. At a concentration of 2.5 M, almost no FUS-PepN activity (0.5 %) was detected. Here, no improvement compared to the single enzyme was achieved. However, the application of ethanol is an alternative to ensure microbial stability. The wheat suspension in the previously named study (Merz et al. 2015b) was microbially stable over 36 days at an ethanol concentration of 8 % (w/v). The fusion protein showed a residual activity of 28 % for FUS-PepN and 64 % for FUS-PepX activity at a concentration of 10 % (w/v). Thus, ethanol seems to be a compound to ensure microbial stability in combination with the fusion protein during food protein hydrolysis. Differences were found for FUS-PepN compared to the single enzyme concerning the sensibility against the metal ion-chelating reagent EDTA and the disulfide bridge-reducing reagent β -mercaptoethanol. The FUS-PepN activity was more insensitive against EDTA than the single enzyme, and inactivation was no longer found for β -mercaptoethanol. This indicates that the fusion protein is more stable against influences from the environment and, thus, preferable for application in complex food matrices. Comparable to the previous study from our group (Stressler et al. 2013b), a strong substrate inhibition was detected for FUS-PepN activity using H-Ala-pNA as a substrate. Due to the fact that FUS-PepN was also inhibited by its products (here: released L-Ala; Stressler et al. 2013b), the Enzyme Kinetics Tool of SigmaPlot was not applicable for nonlinear regression fitting. Thus, SigmaPlot was also used for nonlinear regression fitting with the so-called Weibull function, which is applicable for more complex kinetic determination (Murado and Prieto 2013). Comparable values were determined comparing the K_M and K_{IS} values between the FUS-PepN activity and the single enzyme (Stressler et al. 2013b). The K_M value for FUS-PepX, using H-Ala-Pro-pNA as a substrate, was about 20 % compared to the single enzyme. Thus, the kinetic parameters were not negatively influenced by the fusion of the two enzymes. However, it is noteworthy that synthetic substrates were used in both studies and not original peptide substrates. In a previous study, our group showed for single PepX that the kinetic parameters using original peptide substrates were improved compared to H-Ala-Pro-pNA (Stressler et al. 2014). If this is also the case for the fusion protein has to be determined in another study and was not the focus of this report.

Finally, FUS-PepN-PepX was applied to a prehydrolyzed casein solution. Comparable to our previous study (Stressler et al. 2013b), the relative degree of hydrolysis was increased by about 130 %. This demonstrates that the active site of both enzymes in the fusion

protein was accessible and that the synergistic effect of the FUS-PepN and FUS-PepX activity in the fusion protein was present as it was for the single enzymes. Before combining both enzymes into a fusion protein, our group tested the application of PepN/PepX-combi-CLEAs for protein hydrolysis (Stressler et al. 2015). In this study, the relative degree of hydrolysis was only increased by about 52 %, most probably due to the limited accessibility of the casein-derived peptides to the active site.

The resulting hydrolysates in the current study were tested concerning their antioxidative capacity. The application of the fusion protein increased the antioxidative capacity remarkably. This must be caused by the release of antioxidative amino acids and dipeptides. As an example, the height of the tyrosine peak increased notably as seen in Fig. 6b. The strong antioxidative effect of tyrosine was shown in a previously published study (van Overveld et al. 2000). Due to the specificity of PepX, the release of X-Pro dipeptides, these peptides must also be present in this hydrolysate. The antioxidative effect of tyrosine-containing dipeptides was generally described recently (Torkova et al. 2015). The high antioxidative effect was especially shown for the dipeptide YP (Stressler et al. 2013a). The ABTS- IC_{50} value of the final casein hydrolysate obtained was $5.81 \mu\text{g mL}^{-1}$. This value was very low compared to the ABTS- IC_{50} values obtained ($20\text{--}25 \mu\text{g mL}^{-1}$) for casein hydrolysates produced with *Bacillus lentus* alkaline peptidase (BLAP) in an EMR (Eisele et al. 2013).

In conclusion, the fusion protein consisting of the exopeptidases PepN and PepX from *Lb. helveticus* ATCC 12046 exhibited both enzyme activities. The biochemical characterization of the fusion protein showed high potential of FUS-PepN-PepX for food protein hydrolysis. Due to the high temperature stability of both enzymes and the possibility to use ethanol as a microbial hurdle, the application of the fusion protein in an EMR seems preferable in the future. Due to the high molecular mass of the fusion protein, it should not be able to penetrate the membrane. However, the applicability of the fusion protein in a continuous food protein hydrolysis process, such as an EMR, has to be verified in a further study. The resulting hydrolysate obtained in a batch process exhibited a high degree of hydrolysis, and most likely a reduced bitterness. In addition, the hydrolysate showed the benefit of a high antioxidative capacity and is, therefore, useful as an additive for product protection against oxidative stress.

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Compliance with ethical standards

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no competing interests.

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