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Article

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Salt Taste Enhancing L-Arginyl Dipeptides from Casein and Lysozyme Released by Peptidases of Basidiomycota

Lisa Harth[1]; Ulrike Krah[2]; Diana Linke[*1]; Andreas Dunkel[2]; Thomas Hofmann[2]; Ralf G. Berger[1].

[1]*Institut für Lebensmittelchemie, Leibniz Universität Hannover, Callinstraße 5, 30167 Hannover, Germany*

[2]*Lehrstuhl für Lebensmittelchemie und molekulare Sensorik, Technische Universität München, Lise-Meitner-Straße 34, 85354 Freising*

[*]*Author to whom correspondence should be addressed: Diana Linke, Tel.: +49 511 4581; Fax: +49 511 4547; E-mail address: Diana.Linke@lci.uni-hannover.de*

Abstract

Some L-Arginyl dipeptides were recently identified as salt taste enhancers, thus opening the possibility to reduce the dietary sodium uptake without compromising on palatability. A screening of 15 basidiomycete fungi resulted in the identification of five species secreting a high peptidolytic activity (>3 kAU/mL; azocasein assay). PFP-LC-MS/MS and HILIC-MS/MS confirmed that L-arginyl dipeptides were liberated, when casein or lysozyme served as substrates. Much higher yields of dipeptides (42-75 μmol/g substrate) were released from lysozyme than from casein. The lysozyme hydrolysate generated by the complex set of peptidases of *Trametes versicolor* showed the highest L-arginyl dipeptide yields and a significant salt taste enhancing effect in a model cheese matrix and in a curd cheese. With a broad spectrum of novel specific and non-specific peptidases active in the slightly acidic pH range, *T. versicolor* might be a suitable enzyme source for low-salt dairy products.

Keywords basidiomycota; casein; lysozyme hydrolysis; salt taste enhancers; L-arginyl dipeptides.

Introduction

Protein hydrolysates and purified functional peptides are of increasing interest to the food industry. The enzymatic hydrolysis of abundant milk protein fractions and the characteristics of the peptides obtained were subject of numerous studies.[1-3] These focused on techno-functionalities, such as solubility, emulsifying or foaming properties, and on bioactivities, such as antimicrobial, antiviral, antioxidant, antihypertensive, antithrombotic and taste activities.[2-7] Protein hydrolysis during the fermentation of meat, fish, milk and others is used since centuries to generate attractive savory aromas in food and to concertedly produce seasonings, such as soy or fish sauces.[8, 9] While L-glutamate was recognized as the key umami molecule and taste enhancer almost a century ago,[5] a series of kokumi taste enhancing *γ*-glutamyl dipeptides[10] and salt enhancing L-arginyl dipeptides[6] have just recently been reported, thus opening a new way for highly palatable, but sodium reduced food products.

The limitation of sodium intake has become a worldwide public health care issue, as evidence from epidemiological, intervention, migration, animal and meta-analytical studies indicated a correlation of high sodium intake with hypertension, cardiovascular diseases, stroke and diet-associated diseases for a group of genetically pre-disposed persons.[11-15] Currently, the typical daily sodium intake is more than twice as high as recommended by the World Health Organization (less than 2 grams of sodium or 5 grams of NaCl per day[16]).

Salt taste enhancing peptides would offer an option to reduce the sodium chloride content of savory foods without impairing taste quality and consumer acceptance of the products. Known salt substitutes, such as alkali or earth alkali salts (e. g. KCl, $CaCl_2$, $MgCl_2$) and salt taste enhancers such as L-lysine hydrochloride, L-arginine, L-ornithyl-*β*-alanyl hydrochloride, L-ornithyl-tauryl hydrochloride, trehalose, *N*-geranyl cyclopropyl-carboximide, are all either associated with off-flavors or lack effectiveness for food applications.[17-21] In contrast, the salt taste enhancing L-arginyl dipeptides RA, AR, RG, RS, RV, VR and RM did not show any off-

flavor in aqueous solution.[6] While organic peptide synthesis requires tedious protecting group chemistry, the controlled enzymatic release of such L-arginyl dipeptides from food proteins appears to be a food-grade route to salt taste enhancing peptide mixtures. The objectives of this study were to characterize extracellular mixtures of endo- and exopeptidases in the culture medium of basidiomycetes, to determine their potential in generating L-arginyl dipeptides (RDP) from casein and lysozyme, and to evaluate the hydrolysates' salt taste enhancing activity in model applications.

Materials and Methods

Chemicals

All chemicals and solvents were obtained in the required purity from Sigma-Aldrich (Taufkirchen, Germany), Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany) and VWR International GmbH (Darmstadt, Germany). The substrate lysozyme (type c) from chicken egg white was from Fluka (Seelze, Germany), and casein (a mixture of α-, β- and κ-casein) was from Carl Roth. Gluten was from Nestlé Product Technology Centre (Singen/Hohentwiel, Germany). Soy protein was from LSP Sporternährung (Bonn, Germany), and pea and rice proteins from Bioticana (Rendswühren, Germany). LC-MS/MS reference compounds were: RA, RR, RD, ER, RE, RQ, GR, RG, RH, HR, IR, RI, LR, KR, RK, RM, MR, FR, PR, SR, RS (Bachem AG, Bubendorf, Switzerland); RN, DR, RC, TR, WR, YR, RY, VR, RV (EZBiolab Inc, Carmel, USA); L-isoleucine, L-leucine, L-phenylalanine, L-proline, L-tyrosine (Sigma-Aldrich); L-histidine (Riedel-de Haen, Seelze, Germany); L-tryptophan, L-valine (Merck, Darmstadt, Germany). Stable isotope-labeled amino acids were from Cambridge Isotope Laboratories (Andover, MA, USA). Water used for chromatography was prepared with a Milli-Q Gradient A 10 system (Millipore, Schwalbach, Germany). For sensory analysis, L- alanine, monosodium L-aspartate monohydrate, monosodium L-glutamate

monohydrate, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-tyrosine (Sigma Aldrich); L-lysine monohydrochloride, L-tryptophan, L-valine (Merck) were used.

Strains

15 basidiomycetous strains were used in this study: *Agaricus bisporus* (DSMZ, No. 3054, Abi), *Fistulina hepatica* (DSMZ, No. 4987, Fhe), *Fomitopsis pinicola* (DSMZ, No. 4957, Fpi), *Gloeophyllum odoratum* (CBS, No. 444.61, God), *Grifola frondosa* (CBS, No. 480.63, Gfr), *Hirneola auricula-judae* (DSMZ, No. 11326, Haj), *Lepista nuda* (DSMZ, No. 3347, Lnu), *Meripilus giganteus* (DSMZ, No. 8254, Mgi), *Phanerochaete chrysosporium* (DSMZ, No. 1547, Pch), *Pleurotus eryngii* (CBS, No. 613.91, Per), *Schizophyllum commune* (DSMZ, No. 1024, Sco), *Serpula lacrymans* (CBS, No. 751.79, Sla), *Trametes versicolor* (DSMZ, No. 11269, Tve), *Tremella mesenterica* (DSMZ, No. 1557, Tme) and *Ustilago maydis* (DSMZ, No. 17144, Uma) (Centraalbureau voor Schimmelcultures, CBS, Utrecht, Netherlands and Deutsche Sammlung für Mikroorganismen und Zellkulturen, DSMZ, Braunschweig, Germany).

Cultivation of Basidiomycetes

The strains were maintained on standard nutrient liquid (SNL) agar. SNL agar was prepared on the basis of the Sprecher[22] medium: D-(+)-glucose-monohydrate 30.0 g/L, L-asparagine monohydrate 4.5 g/L, yeast extract 3.0 g/L, KH_2PO_4 1.5 g/L, $MgSO_4$ 0.5 g/L, 15.0 g/L agar agar, 1.0 mL/L trace element solution ($FeCl_3 \cdot 6\ H_2O$ 0.08 g/L, $ZnSO_4 \cdot 7\ H_2O$ 0.09 g/L, $MnSO_4 \cdot H_2O$ 0.03 g/L, $CuSO_4 \cdot 5\ H_2O$ 0.005 g/L, EDTA 0.4 g/L); adjusted to pH 6 with 1 M NaOH before sterilization. Submerged pre-cultures were inoculated with 1 cm² agar plugs with mycelium in 100 mL SNL medium (same medium without agar agar) and homogenized using an Ultra-Turrax (Miccra Art, Müllheim, Germany). Pre-cultures were cultivated for 5-10 days. Subsequently, mycelium of 25 mL of culture liquid was separated by centrifugation

(10 min at 4,800 x g), washed twice with sterile H_2O and transferred into 250 mL minimal medium (D-(+)-glucose-monohydrate 10.0 g/L, yeast extract 1.0 g/L, KH_2PO_4 1.5 g/L, $MgSO_4$ 0.5 g/L, trace element solution (see SNL); adjusted to pH 6 with 1 M NaOH before sterilization). Finally, the cultures were supplemented with 40 g/L dry sterilized substrates (casein, gelatin, gluten, egg white powder, pea, rice or soy proteins; each > 80 % protein). Submerged cultures were kept at 24 °C in a rotary shaker (Infors, Bottmingen, Switzerland) at 150 rpm for up to 16 days. One mL samples were taken every other day, and peptidase activity of the culture supernatant was determined using the azocasein assay. On the day of maximum peptidolytic activity, the cultures were harvested by centrifugation (9,000 x g at 4 °C for 30 min). The supernatants were filtrated, concentrated about 6-fold (10 kDa molecular mass cut off, Sartocon Slice PESU Cassette, Sartorius, Göttingen, Germany) and stored at -20 °C.

Measurement of Peptidase Activity

The azocasein assay of Iversen and Jørgensen was slightly modified.[23] 100 µL substrate (5 % azocasein in H_2O), 375 µL buffer (0.1 M K_2HPO_4/KH_2PO_4 pH 6) and 25 µL sample were mixed and incubated for 20 min at 43 °C in a rotary shaker (Thermomixer, Eppendorf, Hamburg, Germany) at 700 rpm. The reaction was stopped with 1 mL trichloroacetic acid (3 % TCA). For the blanks, the enzyme sample was added after TCA. Samples and blanks were subsequently stored on ice for 10 min and centrifuged at 15,000 g and 20 °C for 15 min. Absorbance of the supernatants was measured at 366 nm using a spectrophotometer (UV-1650 PC, Shimadzu, Duisburg, Germany). One arbitrary Unit (AU) was defined as the enzyme activity that increased the absorbance by 0.01 per min at 43 °C.

Hydrolysis of Synthetic Peptide Substrates

The concentrated culture supernatants were tested against different chromogenic peptide substrates (Arg-*para*-nitroanilide (*p*NA), Gly-Arg-*p*NA and Benzoyl-Arg-*p*NA from Bachem AG (Bubendorf, Switzerland)). The activity assay was performed in 96-well microtiter plates. 20 µL enzyme preparation, 10 µL substrate (2 mM in dimethyl sulfoxide) and 120 µL buffer (50 mM NaAc pH 4, 50 mM K_2HPO_4/KH_2PO_4 pH 6 and 50 mM Tris pH 8) were mixed. The absorption was measured at 37 °C and 405 nm over 60 min in a BioTek Synergy 2™ microtiter plate reader (Bad Friedrichshall, Germany).

Determination of pH Optimum

The pH optima of the peptidase mixtures of different basidiomycetes were determined by azocasein assay as described above but with varying pH in the reaction mixture. Britton-Robinson buffer (40 mM H_3BO_3, 40 mM H_3PO_4 and 40 mM CH_3COOH, titrated to the desired pH with 0.2 M NaOH)[24] was used in the range from pH 2 to 9.

Quantitative Analysis of Soluble Proteins

Soluble protein concentration was measured according to Bradford[25] using the Protein Reagent (No. B6916, Sigma-Aldrich). To determine the increasing solubility of casein during hydrolysis, the casein pellets, after centrifugation at 5,000 g for 30 min, were solubilized in 100 mM NaOH, and the protein concentration was quantified. Casein (0.05-1 mg/mL) in 100 mM NaOH was used as calibration standard.

Preparation of Protein Hydrolysates

Casein and lysozyme were hydrolyzed with peptidase mixtures of the five basidiomycetes Gfr, Pch, Per, Sco and Tve. Reaction mixtures contained 10 mg/mL substrate and an enzyme activity of 400 AU/mg substrate in 50 mM acetate buffer pH 6 in a total volume of 50 mL for human sensory taste evaluation in model cheese matrix. The total volume was four mL for

177 measurements of casein solubility, and one mL for SDS-PAGE analysis and quantification of
178 L-amino acids and L-arginyl dipeptides. Reaction mixtures for human sensory taste evaluation
179 in curd cheese were performed with 20 mg/mL lysozyme, 400 AU/mg lysozyme, pH 5 in a
180 total volume of 25 mL. Blanks were performed with heat-inactivated enzymes and without
181 enzyme addition. After zero, one, five and 24 hours of incubation at 37 °C on a rotary shaker
182 (Thermomixer, Eppendorf, Hamburg, Germany) at 600 rpm, reactions were terminated by
183 heating (99 °C for 30 min for four, 25 and 50 mL samples or ten min for volumes of one mL).
184 Samples were stored at -20 °C. Samples for quantification of L-arginyl dipeptides or human
185 sensory taste evaluation in cheese matrix were freeze-dried and stored at -20 °C. Samples for
186 human sensory evaluation in curd cheese were used directly after inactivation.
187
188 **Determination of the Degree of Protein Hydrolysis**
189 Released L-amino acids [mM/g substrate] in hydrolysates were quantified using *o*-
190 phthalaldehyde (*o*PA) pre-column derivatization, RP-HPLC and fluorescence detection
191 (Shimadzu RF-10AxL, Duisburg, Germany; $\lambda_{excitation}$ = 330 nm, $\lambda_{emission}$ = 460 nm). For *o*PA
192 derivatization, 100 µL 0.5 M borate buffer pH 10, 20 µL *o*PA-reagent (100 mg *o*PA, 1 mL
193 borate buffer, 9 mL MeOH, and 100 µL 3-mercaptopropionic acid), and 10 µL diluted sample
194 were mixed. After 2 min, the reaction was stopped with 50 µL of 1 M CH_3COOH. HPLC was
195 performed on a Nucleodur C18 Pyramid column (250 mm x 4 mm, 5 µm, Macherey-Nagel,
196 Düren, Germany). A flow rate of 1 mL/min and the following gradient of MeOH (eluent A)
197 and 0.1 M sodium acetate containing 0.044 % trimethylamine (adjusted to pH 6.5 with
198 CH_3COOH; eluent B) was used: 0-5 min 10 % A, 5-8 min 15 % A, 8-40 min 60 % A, 40-
199 50 min 100 % A, 50-55 min 10 % A and 55-60 min 10 % A. Measurements were made in
200 duplicates, and concentrations of L-amino acids were calculated using five or six point
201 calibration curves (5 to 100 µM) for each L-amino acid. L-proline, hydroxyproline and L-
202 cysteine were not detectable.

The degree of hydrolysis (DH in %) was calculated after Nielsen: DH [%] = $h/h_{tot} \times 100$ % where h is the concentration of L-amino acids per gram substrate after enzymatic hydrolysis and h_{tot} is the concentration of L-amino acids per gram substrate after total hydrolysis with 6 M HCl at 100 °C for 24 hours.[26]

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analyses were performed according to Laemmli,[27] using 12 and 18 % (w/v) polyacrylamide gels, respectively. Samples were diluted 1:2 with denaturing loading buffer (0.15 M Tris/HCl pH 6.8, 0.2 M DTT, 4 % SDS, 20 % glycerol, 0.2 % bromophenol blue) and incubated at 95 °C for 10 min. After electrophoresis at 15 mA per gel, gels were stained with Instant Blue (Expedeon, Cambridgeshire, Great Britain). For molecular mass determination, marker proteins from 10 to 250 kDa (Precision Plus Protein Standard™, Bio-Rad, München, Germany) or from 1 to 26.6 kDa (Ultra-low Range Marker, Sigma-Aldrich) were used.

Zymography

For semi-native PAGE, 12 % (w/v) polyacrylamide gels containing 1 mg/mL casein or lysozyme as substrates were prepared. Peptidase samples were mixed 1:2 with native loading buffer (like denaturing loading buffer but without DTT) and applied on pre-cooled zymography gels. Electrophoresis was carried out at 4 °C with pre-cooled running buffer at 10 mA per gel. After separation, gels were washed with 2.5 % Triton X-100 and twice with H_2O for 10 min each. After incubation at 20 °C for 16 hours in 100 mL 100 mM phosphate buffer pH 6, zymography gels were then stained with Coomassie Brilliant Blue G-250. Endopeptidases appeared as white bands on blue background.

Quantitation of Bitter-Tasting L-Amino Acids in Protein Hydrolysates

Bitter-tasting amino acids (P, H, L, I, W, Y, F, and V) were quantified by stable isotope dilution analysis by means of HPLC−MS/MS following standard protocols.[28] The measuring system was based on an API 3200 TripleQuad (AB Sciex, Darmstadt, Germany), which was coupled to an HPLC-system of Dionex HPLC UltiMate® 3000 (Dionex, Idstein, Germany). The mass spectrometer with unit mass resolution was operated in the ESI^+ mode with nitrogen (1.7 bar) as curtain gas, zero grade air (3.1 bar) as nebulizer gas. The scan mode was multiple reaction monitoring (MRM). The measuring system was equipped with a 150 mm × 2.0 mm i.d., 5 µm, TSKgel Amide-80 column (Tosoh Bioscience, Stuttgart, Germany) using the chromatographic conditions and ESI^+ instrument settings published recently.[28] For LC-MS/MS analysis of L-amino acids and L-arginyl dipeptides, lyophilisized hydrolysates were diluted by adding 990 µL water and 10 µL isotope-labeled amino acids (1 mg/L, each) as internal standard solution.

Quantitation of L-Arginyl Dipeptides in Protein Hydrolysates

For the analysis of L-arginyl dipeptides, a Dionex HPLC UltiMate® 3000 HPLC system (Dionex, Idstein, Germany), operated with two chromatographic set-ups (system I and II), was hyphentated with an API 4000 QTRAP mass spectrometer (AB Sciex, Darmstadt, Germany) as reported recently.[6] The dipeptides RA, AR, RG, GR, RS, SR, RD, DR, RQ, QR, RK, KR, RE, ER, RF, FR, RT, TR, RN, NR, RW, and WR were analyzed using the chromatographic system I consisting of a 150 mm x 2 mm, 3 µm TSKgel Amide-80 column (Tosoh Bioscience) operated with a flow rate of 0.2 mL/min and the following gradient of eluent A (acetonitrile/ 5 mM ammonium acetate buffer, pH 3.5; 95/5, v/v) and eluent B (5 mM ammonium acetate buffer, pH 3.5): 0-6 min 5 % B, 25 min 30 % B, 40 min 100 % B, 45-60 min 5 % B. Analysis of the dipeptides RP, PR, RV, VR, RM, MR, RR, IR, RI, RL, LR, RY, and YR was done using chromatographic system II consisting of a 150 mm x 2 mm, 3 µm Luna PFP column (Phenomenex, Aschaffenburg, Germany) operated with a flow rate of

254 0.2 mL/min and the following gradient of eluent A (acetonitrile with 1 % formic acid) and
255 eluent B (1 % formic acid): 0-6 min 100 % B, 10 min 90 % B, 14-19 min 0 % B, 21-30 min
256 100 % B. In both systems, a sample aliquot of 2 µL was injected. The mass spectrometer
257 with unit mass resolution was operated in the ESI^+ mode with nitrogen (1.7 bar) as curtain
258 gas, zero grade air (3.1 bar) as nebulizer gas. The scan mode was multiple reaction monitoring
259 (MRM). For MS conditions see supporting info of Schindler *et al.* (2011).[6] Data2 processing
260 operations were carried out by Analyst 1.5 (AB Sciex, Darmstadt, Germany). Quantitative
261 analysis was performed by means of external standard calibration with 1:10, 1:20, 1:50,
262 1:100, 1:200, 1:500, and 1:1000 dilutions of an aqueous stock solution containing RDP
263 (50 mg/L).
264
265 **Preparation of a Cheese Taste Matrix**
266 To evaluate the salt taste enhancing activity of hydrolysates, a cheese taste matrix was
267 prepared by mixing all key taste compounds recently identified in a Gouda cheese, each in its
268 natural concentration.[29] To achieve this, L-lysine (742.8 mg/L), monosodium L-glutamate
269 monohydrate (572.0 mg/L), L-leucine (505.6 mg/L), L-phenylalanine (255.2 mg/L), L-tyrosine
270 (180.8 mg/L), L-isoleucine (166.4 mg/L), L-valine (132.9 mg/L), L-methionine (81.1 mg/L),
271 L-alanine (77.3 mg/L), monosodium L-aspartate monohydrate (62.4 mg/L), L-tryptophan
272 (38.3 mg/L), sodium chloride (2460.0 mg/L), potassium dihydrogen phosphate (569.3 mg/L),
273 lactate (1480.5 mg/L), calcium acetate (372.0 mg/L) and magnesium chloride (582.5 mg/L)
274 were dissolved in water (Evian, Danone, Wiesbaden, Germany), followed by an adjustment of
275 the pH value to 5.7 by titrating with calcium hydroxide.
276
277 **Human Sensory Taste Evaluation**
278 Sixteen healthy panelists with no history of known taste or smell disorders had given
279 informed consent to participate in the sensory tests and were trained in the sensory evaluation

of aqueous solutions of standard taste compounds: [28, 30, 31] sucralose (1 - 5030 µmol/L) for sweet taste, monosodium L-glutamate (1 - 60 mmol/L) for umami taste, caffeine (0.1 - 10 mmol/L) for bitter taste, citric acid (1 - 80 mmol/L) for sour taste, and NaCl (4 - 110 mmol/L) for salty taste. Having participated in sensory experiments on a regular basis for at least one year, the panelists were accustomed to the techniques applied.

First, a selection of lysozyme hydrolysates (Gfr, Pch, Per, Sco, and Tve, 24 h incubation; 0.4 % each) was dissolved in the cheese matrix and then sensorially evaluated by means of a profile sensory test. The intensities of the basic taste modalities salty, sweet, umami, bitter, and sour were assessed on a scale of 0 (not perceivable) to 5 (strongly perceivable).

To test the ability of the sensory panel to differentiate different sodium levels in the cheese taste matrix, each panelist was asked to arrange three-digit random-coded test samples, containing sodium in concentrations varying between 40 and 60 mM sodium (in 10.0, 5.0, 2.5 mM steps), according to the perceived salt intensity in the cheese matrix. The so-called Friedman value determined for each panelist and calculated as ranking sum of each sample.

For the determination of the salt taste enhancing (STE) activity of protein hydrolysates, a two alternative forced choice (2-AFC) test was performed. Two solutions of the cheese taste matrix solution (50 mM sodium), one without and one with protein hydrolysate (0.5 %, w/v) added, were randomly presented to the assessors who were asked to identify the sample showing the higher salt taste intensity.

In addition, a 2-AFC test was performed using low-fat (0.3 %) curd cheese as matrix. Two samples of the curd cheese, one with water (1 mL/g) and one with hydrolysate (1 mL/g (≙ 0.5 %, w/v)) added, each with 50 mM NaCl, were randomly presented to the assessors who were asked to identify the sample showing the higher salt taste intensity.

Identification of Peptidases

For the identification of peptidases from Tve the concentrated culture supernatant was partially purified using ion chromatography. Five mL of the sample were diluted with 40 mL running buffer (20 mM Tris pH 7.5), membrane filtered (0.45 µm), and applied to a pre-equilibrated HiTrap Q XL (1 mL, GE Healthcare, München, Germany), washed with 50 mL running buffer and eluted with elution buffer (20 mM Tris + 1 M NaCl pH 7.5) in a linear 20 mL gradient. Fractions of 1 mL were collected at a flow rate of 1 mL/min, fivefold concentrated, desalted by ultrafiltration (MWCO 10 kDa), and analyzed by semi-native SDS-PAGE and zymography. Peptidase bands were cut out of the SDS-gel and hydrolyzed by trypsin according to standard protocols. For peptide mass fingerprinting, the amino acid sequences of tryptic peptides of peptidases were deduced by ESI-MS/MS mass spectra using a maXis QTOF mass spectrometer (Bruker, Bremen, Germany) and the Mascot search algorithm together with the NCBI in-house database. A minimum Mascot score of 100 was chosen for reliable identifications as described in detail elsewhere.[32]

Results and Discussion

Peptidolytic Activity of Basidiomycetes

15 basidiomycetes were submerged cultivated in minimal medium with gluten as major carbon and nitrogen source to stimulate the secretion of peptidases (Table 1). The proline-rich gluten was chosen because it proved to be a potent substrate for inducing high peptidolytic activities in previous studies.[33, 34] After a cultivation time of 15 days, the fungal strains reached different maximal peptidolytic activities from less than 0.2 kAU/mL to 6.3 kAU/mL. Maximal activities were reached after 6 to 11 days (Table 1). Highest peptidase activities were found in the supernatant of *Phanerochaete chrysosporium* with 6.3 kAU/mL and *Trametes versicolor* with 5.7 kAU/mL, respectively. The five most active basidiomycetes

(Gfr, Pch, Per, Sco and Tve; activity > 3 kAU/mL) hydrolyzed both, casein and lysozyme, as was shown by zymography (Figure 1) and SDS-PAGE (Figure 2). Hence, they were thought to be suitable for applications in food, with the aim of generating casein and lysozyme hydrolysates with functional peptides, such as L-arginyl dipeptides.

Cultivation with Different Protein Substrates

Peptidases cutting next to arginine are of particular interest for the release of STE arginyl-peptides. In order to examine inducer properties of the protein substrate, the five most active basidiomycetes (Gfr, Pch, Per, Sco and Tve) were cultivated with storage protein mixtures from pea, rice, gelatin and soy. These proteins were chosen due to their higher arginine contents compared to gluten (see Table 2). However, the secretion pattern of peptidases did not change in the zymography (data not shown). None of the basidiomycetes showed arginine-specific endo-, dipeptidyl- or exopeptidase activity as determined using the artificial substrates Bz-Arg-*p*NA, Gly-Arg-*p*NA and Arg-*p*NA. Although the degradation pattern in the zymography did not change, overall activities varied strongly, depending on the protein substrate used (Table 3). For Tve the activity was enhanced from 5.8 kAU/mL to 15 kAU/mL when pea proteins were used instead of gluten, and by a factor of 78 in comparison to SNL medium without protein substrate. Rice proteins were most effectively for Per and Sco, pea proteins for Pch and Tve, while gluten was best for inducing peptidolytic activity of Gfr. Consequently, no general ranking for effective proteins substrates was obtained from the data. Egg white and milk proteins were the intended precursor proteins for the generation of STE peptides in dairy products. When Tve, one of the most active candidates, was supplied with these substrates, fungal growth was slow, and peptidolytic activity (up to 6 kAU/mL) was detected towards the end of the cultivation only (day 16, Table 3).

pH Optima of the Peptidases

As a synergistic action of different peptidases favors extensive hydrolysis, [35] it was intended to use concentrated culture supernatants with their complex sets of (exo- and endo-)peptidases to generate the target STE peptides. Gfr, Pch, Per, Sco and Tve, which exhibited highest extracellular peptidase activities (Table 3), were used for detailed studies. Maximal enzyme activities of Per were detected at pH 6, while Gfr, Pch, Sco and Tve showed highest activities at pH 5 (Figure 3). All of the five peptidase mixtures were active in a broad and slightly acidic pH range and should, thus, be applicable in fermented dairy products, too. The complexity of the peptidase mixture with up to six clearly visible peptidase bands in the casein zymography may have contributed to the broad pH activity range (Figure 1).

Enzymatic Degradation of Casein and Lysozyme

The peptidases of the five selected basidiomycetes hydrolyzed casein and lysozyme, as was demonstrated by the analysis of released amino acids, SDS-PAGE and zymography. Moreover, zymography (Figure 1) indicated that the peptidolytic activity of most basidiomycetes was composed of several visible endopeptidases, at least two (Tve) and up to six (Pch) enzymes. The copolymerized substrates casein or lysozyme led to bands with different molecular masses and varying band intensities highlighting the individual substrate specificity of the peptidases (Figure 1). In the case of Pch, for example, six bands were visible in the casein zymography and only one weak band in the lysozyme zymography. Furthermore, the degree of hydrolysis (DH) was determined after zero, one, five and 24 hours of enzymatic hydrolysis of casein (Table 4a) and lysozyme (Table 4b). The DH increased significantly with extended incubation times and amounted to 14-29 % after 24 hours depending on enzyme mix and substrate. A high DH analytically represents a high concentration of free L-amino acids and is a proof for exopeptidase activity in addition to the endopeptidases detected in the zymography. For both substrates, similar DH were obtained after 24 hours of hydrolysis for Gfr (16 %), Sco (21-22 %) and Tve (18 %), respectively. In contrast, Pch and Per hydrolyzed

casein more efficiently (29 % and 19 %, respectively) than lysozyme (16 % and 19 %, respectively). Moreover, both fungi showed significantly more endopeptidase bands when using casein instead of lysozyme as copolymerized substrate in the zymography.

After 24 hours of hydrolysis, the released L-amino acids accounted for 1.1-2.0 mmol/g casein and 1.0-1.8 mmol/g lysozyme. The main L-amino acids of casein hydrolysates generated with the peptidases of Gfr, Pch, Per and Sco were L-lysine, L-leucine and L-glutamic acid, each with 117-257 µmol/g substrate. These L-amino acids represent the three major amino acids of casein. For lysozyme hydrolysates generated with the peptidases of Gfr, Per, Sco and Tve L-arginine, L-lysine, L-leucine and L-alanine were most predominant, with 85-258 µmol/g substrate. Apart from L-lysine, these amino acids again represent the main constituents of the substrate. These data suggested that the free amino acids resulted from a non-specific hydrolysis. In contrast, casein hydrolysis with Tve liberated L-phenylalanine above the expected level, and Pch predominantly released L-glutamic acid from lysozyme indicating the presence of peptidases with preferred cleavage specificity.

The composition of the hydrolysates was visualized using SDS-PAGE (shown for Tve in Figure 2). Among the five basidiomycetes, there were nearly no differences in protein patterns after casein hydrolysis. For all basidiomycetes, the soluble casein bands disappeared completely within one hour of hydrolysis, but insoluble casein pellets remained (see section below). No new bands were visible above 10 kDa. For lysozyme as the substrate, new bands appeared between 3.5 and 14 kDa. There were varying peptide patterns between the various basidiomycetes, pointing out the different endo-specificities. After 24 hours, there was still intact lysozyme left in the case of Pch, Sco and Tve.

Increase of Casein Solubility

Protein solubility, an important requirement for functional and bioactive properties, was improved by peptidolysis, [36] as was also demonstrated along the way in the present study.

Hydrolyses were performed at pH 6, where casein is poorly soluble. The residual insoluble pellet decreased with prolonged incubation time. After 24 hours of hydrolysis 88-97 % of the initial insoluble pellets were hydrolyzed by each basidiomycetous peptidase mixture (Table 4), similar to casein hydrolysis with the commercial peptidases papain, pancreactin and trypsin.[3, 36] Especially for Pch, Per, Sco and Tve the increase in solubility was larger than 50 % after five hours, whereas Gfr increased solubility slowly. After five hours, still more than 90 % of the initial pellet remained insoluble. These findings suggest that the peptidases of Gfr were less suitable for heterogeneous catalysis.

Quantitation of L-Arginyl Dipeptides in Protein Hydrolysates

For the quantification of released arginyl dipeptides (RDPs), the hydrolysates of lysozyme (Figure 2a, Table 6a) and casein (Figure 2b, Table 6b) prepared by incubation with Gfr, Pch, Per, Sco and Tve, respectively for 24 h, were analyzed by PFP-LC-MS/MS and HILIC-MS/MS, respectively. 14 RDPs, namely RP, RA, AR, RG, RS, RV, VR, RM, RR, RD, MR, RQ, RK, and KR, were recently reported to exhibit salt taste enhancement.[6] Accordingly, the concentration of STE-active RDPs in the hydrolysates was calculated as the sum of RP/PR, RA/AR, RG/GR, RS/SR, RV/VR, RM/MR, RR, RD/DR, RQ/QR, and RK/KR to approximate the STE-activity of the hydrolysates.

In general, RDPs were released during all hydrolyses. The yields of RDPs ranged from 43.2 to 74.9 µmol/g for lysozyme (Table 4a), and from 1.3 to 3.6 µmol/g for casein (Table 4b). The substrate lysozyme led to much higher yields of RDP for all tested basidiomycetes, at least partly caused by its higher arginine contents compared to casein (see Table 2). The enzymatically released yields of RDPs were between 5 to 10.2 % (Tve) of the maximal theoretical yield.

Among the STE-active RDPs, the highest concentration of 47.6 µmol/g was observed when lysozyme was hydrolyzed using Pch (Table 4a). Furthermore, the LC-MS/MS analysis of

RDPs in lysozyme hydrolysates indicated particularly for Gfr, Pch, Sco, and Tve the presence of salt taste enhancing dipeptides, such as RG and RS in yields of 10 - 22 µmol/g lysozyme hydrolysate (Figure 4, Table 6a). Several salt taste inhibiting RDPs were also released from lysozyme, for example RH and HR by Pch, Per, Sco, and Tve, and RW/WR by Gfr, Per, Sco and Tve, respectively. STE-active dipeptides (RP, RV, VR) were released in yields of 0.2 to 0.4 µmol/g casein, whereas salt taste inhibiting compounds (RJ, JR) were liberated in amounts up to 1.4 µmol/g casein. In summary, LC-MS/MS analysis of the RDP release patterns showed a high similarity between the different peptidase sources, particularly when lysozyme was the substrate.

Sensory Evaluation of Saltiness Enhancement of Lysozyme Hydrolysates

In order to investigate the sensory impact of the increased STE-active RDPs, lysozyme hydrolysates were evaluated by a trained sensory panel of 12 persons who were able to distinguish between 5 mM sodium in a concentration range of 40 to 60 mM sodium. First, the hydrolysates obtained from lysozyme after enzymatic digestion (24 h) with Gfr, Pch, Per, Sco and Tve were evaluated in a cheese taste matrix which was prepared by mixing all key taste-active amino acids, organic acids and minerals, each in its natural concentration as recently determined in a Gouda cheese,[29] and adapted to 50 mM Na^+. Only the Tve hydrolysate of lysozyme revealed a significant salt taste enhancing effect (Table 5). Furthermore, hydrolysates were evaluated in low-fat curd cheese with NaCl adjusted to 50 mM. Both, Tve and Gfr, revealed an impact on the perceived salt taste. The panelists specified hydrolysates from Tve as more effective in salt taste enhancement than Gfr hydrolysates. Considering the higher amounts of STE-active RDPs in the lysozyme hydrolysate treated with Pch (47.6 µmol/g lysozyme) when compared to Gfr and Tve (~25 µmol/g lysozyme), other constituents than just the STE-active RDPs seemed to contribute to the salt taste enhancing effects perceived.

As bitter taste is known to lower perceived saltiness,[6] also the bitter taste intensity of the hydrolysates was sensorially evaluated, and bitter tasting amino acids (P, H, L, I, W, Y, F, and V) were quantitated (Table 5). [37-39] The profile sensory test revealed high bitter scores for three basidiomycetes (Sco, Gfr and Pch), whereas for Per and Tve only slight bitterness was detected. Comparing the results with the sum of bitter amino acids, the bitterness of the lysozyme hydrolysate from Pch with a bitter score of 4.7 is explained by the highest release of bitter amino acids (99.9 μmol/g lysozyme). Additional bitter peptides seem to influence the bitter score of lysozyme hydrolysates of Gfr and Sco, considering that Tve released comparable quantities of bitter amino acids. Next to the salt inhibitory effect of bitter amino acids and peptides, the discrepancy between sensory evaluation and STE-active RDP levels indicated the presence of other, currently unknown STE compounds.

Identification of Peptidases

Several peptidases of the most promising candidate Tve were identified to determine which peptidases might have been involved in the formation of salt taste enhancing hydrolysates (Table S1). The identified enzymes were aspartic A01 peptidases (AC No. EIW62808, EIW63301), a peptidyl-Lys M35 (XP_008032702) and a M36 (EIW51569) metallopeptidase, and serine peptidases of the MEROPS families S28 (EIW65216, EIW61562), S41 (XP_008043737) and S53 (EIW61376, EIW61051, EIW59803). Endopeptidases with a broad cleavage specificity and a preference for hydrophobic amino acids (A01, S53, M36) predominated. They lead to a rather non-specific hydrolysis. However, the peptidases S28, S41 and the tripeptidyl-peptidase S53 are of special interest. S28 peptidases are proline-specific enzymes.[40] For prolyl-peptidases, a debittering effect was described.[41] Also in this work, they may have been responsible for a debittering effect, because Tve hydrolysates were less bitter than most other (Table 5). The S41 peptidase is a C-terminal processing enzyme that recognizes a tripeptide and cleaves at a variable distance.[40] A typical cleavage-site

485 contains Arg in P1' and an aliphatic amino acid in P2'. The S41 peptidase could be involved
486 in the formation of arginyl-peptides. To our knowledge, only one other fungal S41 peptidase
487 has been described in literature.[42] S53 tripeptidyl-peptidases release tripeptides and are
488 involved in the formation of small peptides.[40] To our knowledge, a few of these peptidases
489 were found in fungi, such as *Rhizopus*[43] and *Aspergillus*[44], but not in basidiomycetes.
490 In conclusion, STE peptides were released from food-grade proteins using specific and non
491 specific peptidases of basidiomycetes. Novel customized peptidases as presented in this study
492 appear to be necessary to release STE peptides, such as RDP, without risking a concurrent
493 extensive release of bitter peptides and bitter amino acids.
494

495 Abbreviations

496	Gfr	*Grifola frondosa*
497	Pch	*Phanerochaete chrysosporium*
498	Per	*Pleurotus eryngii*
499	Sco	*Schizophyllum commune*
500	Tve	*Trametes versicolor*
501	RDP	L-arginyl dipeptides
502	STE	salt taste enhancing
503	DH	degree of hydrolysis
504	J	Isoleucin, Leucin

505

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Notes

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Figure captions

Fig. 1: Zymography with extracellular basidiomycetous peptidases. Casein (left) and lysozyme (right) were copolymerized. *Grifola frondosa* (Gfr), *Phanerochaete chrysosporium* (Pch), *Pleurotus eryngii* (Per), *Schizophyllum commune* (Sco) and *Trametes versicolor* (Tve). M – Marker (Precision plus Protein™ Standard). All fungi showed a variety of extracellular endopeptidases, visible as white bands.

Fig. 2a and b: SDS-PAGE analysis of the casein and lysozyme hydrolysates produced by peptidases of *Trametes versicolor*. The substrates casein (a, 12 % acrylamide) and lysozyme (b, 18 % acrylamide) were hydrolyzed for 0-24 h. M – Marker (Precision Plus Protein™ Standard, Ultra-low Range Marker).

Fig. 3a and b: Effect of pH on the activity of basidiomycete peptidases. pH optima of basidiomycete peptidases were determined in Britton-Robinson buffer (pH 2 to 9; azocasein assay). Relative enzyme activity [%] was defined as the percentage of activity detected with respect to the maximum observed peptidase activity for each basidiomycete in the experiment. Values are the average of triplicate experiments, with standard deviation shown as error bars. *Grifola frondosa* (Gfr), *Phanerochaete chrysosporium* (Pch), *Pleurotus eryngii* (Per), *Schizophyllum commune* (Sco) and *Trametes versicolor* (Tve).

Fig. 4: Concentrations of L-arginyl dipeptides in hydrolysates of lysozyme and casein from selected peptidases. *Grifola frondosa* (Gfr), *Phanerochaete chrysosporium* (Pch), *Pleurotus eryngii* (Per), *Schizophyllum commune* (Sco) and *Trametes versicolor* (Tve).

654 # Tables

655 Table 1: Extracellular peptidase activity of basidiomycetes submerged cultured with gluten.

Basidiomycete	abbreviation	peptidase activity [AU/mL]		maximal activity [d]
Phanerochaete chrysosporium	Pch	6294	± 127	7
Trametes versicolor	Tve	5766	± 76	9
Schizophyllum commune	Sco	4014	± 59	8
Grifola frondosa	Gfr	3654	± 110	7
Pleurotus eryngii	Per	3276	± 119	7
Tremella mesenterica	Tme	1860	± 170	10
Ustilago maydis	Uma	1716	± 51	8
Fomitopsis pinicola	Fpi	1578	± 8	10
Meripilus giganteus	Mgi	1482	± 178	10
Hirneola auricula-judae	Haj	966	± 76	11
Lepista nuda	Lnu	792	± 51	6
Serpula lacrymans	Sla	216	± 17	8
Fistulina hepatica	Fhe	< 200	-	-
Gloeophyllum odoratum	God	< 200	-	-
Agaricus bisporus	Abi	< 200	-	-

656 * Data expressed as mean ± standard deviation of two replicates.

657

658 Table 2: L-Arginine in different protein substrates. Proteins extracted from wheat, soybean,
659 rice and pea were used.

	arginine [%]*
casein	3,7
wheat, whole grain	4,5
soy, dry seeds	6,8
rice, unpolished	7,1
gelatin	7,6
lysozyme	12,1
peas, dry seeds	14,3

660 * Arginine share of total amino acids [mg/g].[45]

661

662 Table 3: Extracellular peptidase activity of basidiomycetes submerged cultured with different
663 substrates. (SNL and MM without protein substrates.)

basidiomycete	abbreviation	substrate	maximal peptidase activity [AU/mL]		maximal activity [d]
Grifola frondosa	Gfr	gluten	3654	± 110	7
		pea protein	< 200	-	-
		rice protein	1776	± 153	8
		soy protein	420	± 136	8
Phanerochaete chrysosporium	Pch	gluten	6294	± 127	7
		pea protein	13200	± 339	8
		rice protein	3462	± 25	8
		soy protein	6648	± 356	8
Pleurotus eryngii	Per	gluten	3276	± 119	7
		pea protein	4368	± 288	6
		rice protein	5118	± 552	6
		soy protein	1830	± 212	8
		gelatin	< 200	-	-
Schizophyllum commune	Sco	gluten	4014	± 59	8
		pea protein	7908	± 238	8
		rice protein	9132	± 221	8
		soy protein	1986	± 178	8
Trametes versicolor	Tve	gluten	5766	± 76	9
		pea protein	15060	± 1137	8
		rice protein	2184	± 356	4
		soy protein	8898	± 144	8
		SNL	< 200	-	-
		MM	< 200	-	-
		casein	6282	± 76	16
		egg white	3510	± 110	16

664 * Data expressed as mean ± standard deviation of two replicates.

665

666

667 Table 4a: Release of L-arginyl dipeptides from lysozyme by peptidases of basidiomycetes.

	t [h]	DH [%]		c^1 (RDP) [µmol/g substrate]	c^1 (STE RDP) [µmol/g substrate]
Gfr	0	0	-	0.6	0.3
	1	2.3	± 0.2		
	5	5.6	± 0.5		
	24	15.8	± 2.1	43.2	25.0
Pch	0	0	-	0.3	0.1
	1	0.6	± 0.2		
	5	2.3	± 0.1		
	24	16.0	± 0.2	73.2	47.6
Per	0	0	-	0.7	0.4
	1	0.7	± 0.1		
	5	5.8	± 0.1		
	24	14.3	± 0.4	67.2	19.1
Sco	0	0	-	0.5	0.2
	1	1.7	± 0.2		
	5	10.9	± 0.4		
	24	22.0	± 0.6	61.1	35.5
Tve	0	0	-	0.2	0.1
	1	0.8	± 0.2		
	5	5.7	± 0.2		
	24	17.6	± 0.1	74.9	25.7

668 * Abbreviations: DH – degree of hydrolysis, t – incubation time, c^1 (RDP) – sum of released
669 L-arginyl dipeptides, c^1 (STE RDP) – sum of released salt taste enhancing L-arginyl
670 dipeptides.
671
672

673 Table 4b: Release of L-arginyl dipeptides from casein by peptidases of basidiomycetes.

	t [h]	DH [%]		insoluble casein pellet [%]		c (RDP) [µmol/g substrate]	c (STE RDP) [µmol/g substrate]
Gfr	0	0.0	-	100	± 1	1.0	0.2
	1	1.4	± 0.2	99	± 5		
	5	7.5	± 0.7	93	± 4		
	24	15.9	± 0.4	12	± 1	2.1	0.6
Pch	0	0.0	-	100	± 5	0.4	0.1
	1	3.9	± 0.3	76	± 3		
	5	12.5	± 0.4	32	± 3		
	24	29.1	± 0.3	5	± 0	2.4	0.3
Per	0	0.0	-	100	± 5	0.3	0.1
	1	1.5	± 0.0	76	± 3		
	5	4.9	± 0.4	35	± 2		
	24	18.8	± 0.9	3	± 0	2.8	0.5
Sco	0	0.0	-	100	± 6	0.6	0.1
	1	2.9	± 0.6	98	± 4		
	5	9.9	± 0.5	33	± 8		
	24	21.0	± 0.3	6	± 1	1.3	0.4
Tve	0	0.0	-	100	± 1	0.1	n. n.
	1	2.0	± 0.2	58	± 0		
	5	6.0	± 0.1	47	± 2		
	24	17.9	± 0.7	10	± 1	3.6	0.8

674 * Abbreviations: DH – degree of hydrolysis, t – incubation time, c (RDP) – sum of released L-
675 arginyl dipeptides, c (STE RDP) – sum of released salt taste enhancing L-arginyl dipeptides.
676 n. n.: concentration < 0.001 µmol/g substrate.

677

678 Table 5: Salt taste enhancing L-arginyl dipeptides (STE RDP) and bitter amino acids in
679 enzymatic lysozyme hydrolysates in comparison to their sensory characteristics.

	c^{1} (STE RDP) [µmol/g substrate]	c^{1} (bitter amino acids) [µmol/g substrate]	bitter score	sweet score	STE effect (α-level)
Per	19.1	19.8	0.7	3.3	> 0.05
Tve	25.7	59.8	1.8	1.8	0.01
Sco	35.5	55.8	3.5	2.5	> 0.05
Gfr	25.0	45.4	4.0	1.3	> 0.05
Pch	47.6	99.9	4.7	0.5	> 0.05

680 c^{1} (STE RDP) – sum of released salt taste enhancing L-arginyl dipeptides, [1]less blank value,
681 α – level of significance.

682

683

684 Table 6a: Concentration of L-arginyl dipeptides in hydrolysates of lysozyme
685 [µmol/g substrate].

	Gfr	Pch	Per	Sco	Tve
RP/PR	0.367	0.171	0.199	0.344	0.097
RA/AR	0.309	0.372	0.468	0.515	0.364
RG/GR	12.317	22.573	7.330	16.497	12.734
RS/SR	2.763	4.547	1.233	4.019	3.403
RV/VR	0.249	1.229	0.579	0.522	0.534
RM/MR	0.491	0.428	0.058	0.480	0.317
RR	0.641	1.259	0.365	0.408	0.499
RD/DR	7.820	16.541	8.690	12.443	7.453
RQ/QR	0.203	0.502	0.283	0.363	0.393
RK/KR	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
RL/RI/IR	7.728	2.139	2.105	5.714	7.192
RE/ER	0.289	0.688	0.292	0.370	0.372
RY/YR	1.657	2.813	0.461	2.114	0.529
RF/FR	0.036	0.078	0.042	0.135	0.180
RN/NR	2.499	0.806	0.675	2.827	1.051
RH/HR	2.039	16.471	35.612	9.156	30.255
RT/TR	0.612	0.726	0.331	0.897	0.542
RW/WR	2.564	1.017	7.986	3.401	8.255
RC/CR	0.590	0.849	0.451	0.901	0.737

686

687 Table 6b: Concentrations of L-arginyl dipeptides in hydrolysates of casein [µmol/g substrate].

	Gfr	Pch	Per	Sco	Tve
RP/PR	0.232	0.221	0.321	0.048	0.394
RA/AR	0.003	0.002	0.001	0.002	0.007
RG/GR	0.012	0.009	0.006	0.011	0.004
RS/SR	0.100	0.056	0.023	0.086	0.031
RV/VR	0.190	0.024	0.174	0.165	0.416
RM/MR	< 0.001	0.012	0.002	0.003	0.006
RR	< 0.001	< 0.001	< 0.001	0.002	< 0.001
RD/DR	0.029	0.040	0.153	0.013	0.192
RQ/QR	0.051	0.035	0.100	0.032	0.122
RK/KR	< 0.001	0.011	< 0.001	< 0.001	0.004
RL/RI/IR	< 0.001	1.422	1.300	0.085	1.379
RE/ER	0.316	0.072	0.082	0.188	0.616
RY/YR	< 0.001	< 0.001	0.011	< 0.001	< 0.001
RF/FR	0.032	0.102	0.278	0.009	0.101
RN/NR	< 0.001	0.047	< 0.001	< 0.001	0.023
RH/HR	< 0.001	0.016	0.053	< 0.001	0.168
RT/TR	0.175	0.028	0.020	0.115	0.023
RW/WR	0.001	< 0.001	0.001	0.001	< 0.001
RC/CR	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

688

689

690 Supplementary table S1: Identified peptidases of the basidiomycete *Trametes versicolor*.

Peptidase	Accession number	Peptides found	Mascot score	Sequence coverage [%]
Aspartatpeptidase A01	EIW62808	SKYTAASSSTSVKK, YTAASSSTSVKK, LASSGSELYLGGTDSK	496	7
Aspartatpeptidase A01	EIW63301	STTFVQGSR, SGTDTVTVGGVAAK	109	5
Peptidyl-Lys Metallopeptidase M35	XP_008032702	ETYVGCSTSQK, SALTTAAPNALTYATNAK, SYLTANTAATTR, AGTLIHESSHFTK	621	15
Metallopeptidase M36	EIW51569	ASYLVLPITK, YGFTEAAFNFQTNNFGK, MFLWDLTSPQR, SHPYSTSATVNPLR	170	8
Serinpeptidase S28	EIW61562	YWLSDR, QSAPAACITQVER, TIDEVDR, TIDEVDRLITSPNAK, LAYDPGEHFQVPPEPD-VEAVNK, LITSPNAK, ATDLSQTWR, LITQD-YASLICK, LGGYSIAYDR, ADTTLRPFK	452	19
Serinpeptidase S28	EIW61562	YYGLSNPFPDLSVK, FHTIQQAIDDLEYFAK, NTKEIDSIK, LVQPAYDER, EATLAADGTNFR	266	11
Serinpeptidase S41	XP_008043737	TFVPPADALACMK, QNVLDVVSR, SPAPFQ-DSTTNIR, VLAIEGVDPYAYAVK, IAETQSG-NYLDLGVR, VNSAFSSYR, SFILPDKK, SLGS-FQNPGFQSTNR, SSSDNYMSPPSSR, VINGQT-FVESQR, FLDVCPFSVDLPEDPPFDPSK, IALF-GGKPGLATQFK, GMAGNQVLEWFDIDSEIK, TANLKDDPLAPPDLLVSGDFR, IAYSFLDET-LPIEYR, SELPHFR, FAYTADTYNNPQNLWT-FAAK	1784	39
Tripeptidyl-Peptidase A S53	EIW61376	TLAPGTYK, AIPDVAAQADLFR	110	3
Serinpeptidase S53	EIW61051	LANQLCNAYAQLGAR, AGWDPVTGLGTPN-FAK, GTSILFASGDGGVAGSQTSSCTK, LLTAVGL	865	10
Serinpeptidase S53	EIW59803	NSLGVAGYLEEFANR, ADLQTFFSR, TDAV-GGTFTTVR, VGSVGGTSASSPTFAGVIALLN-DFR	822	10

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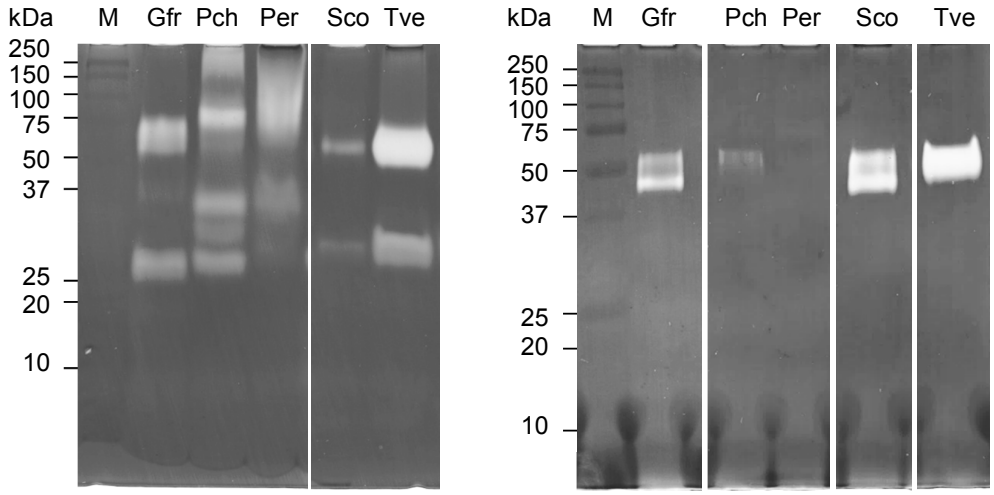
692 **Figures**



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695 Figure 2a:

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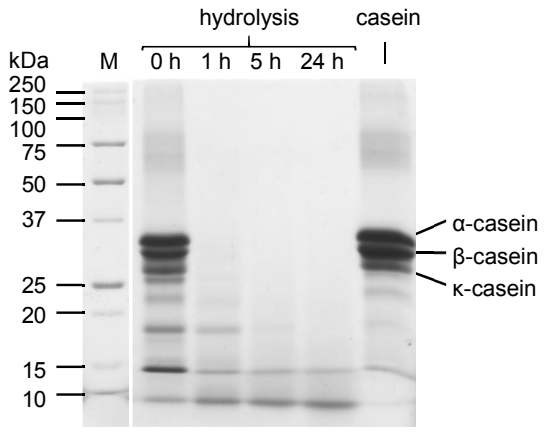

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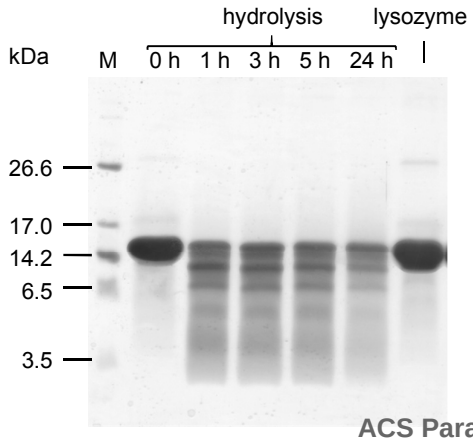

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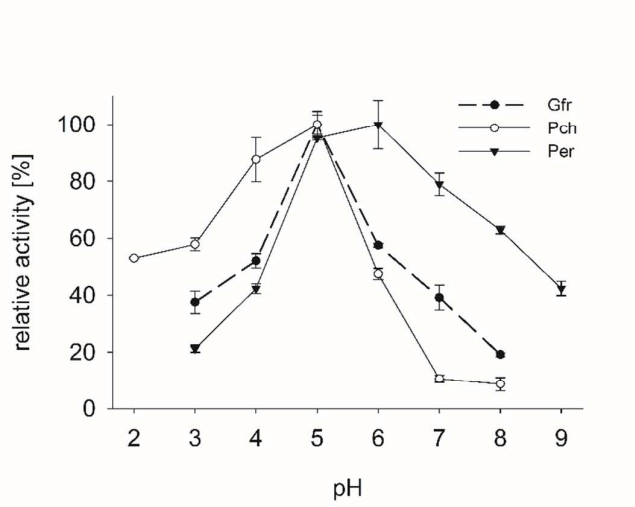

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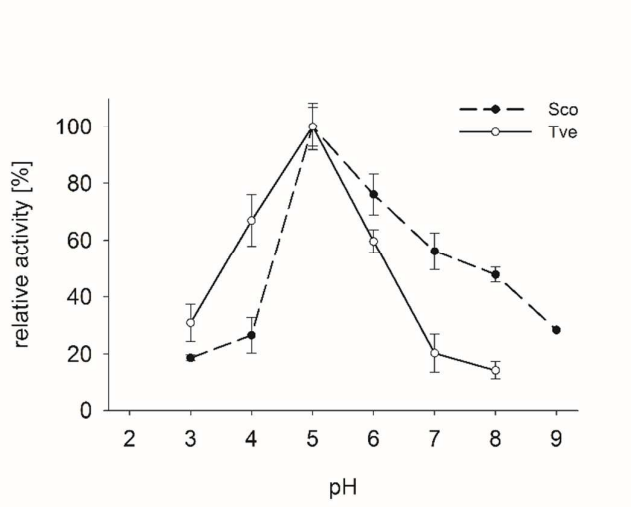

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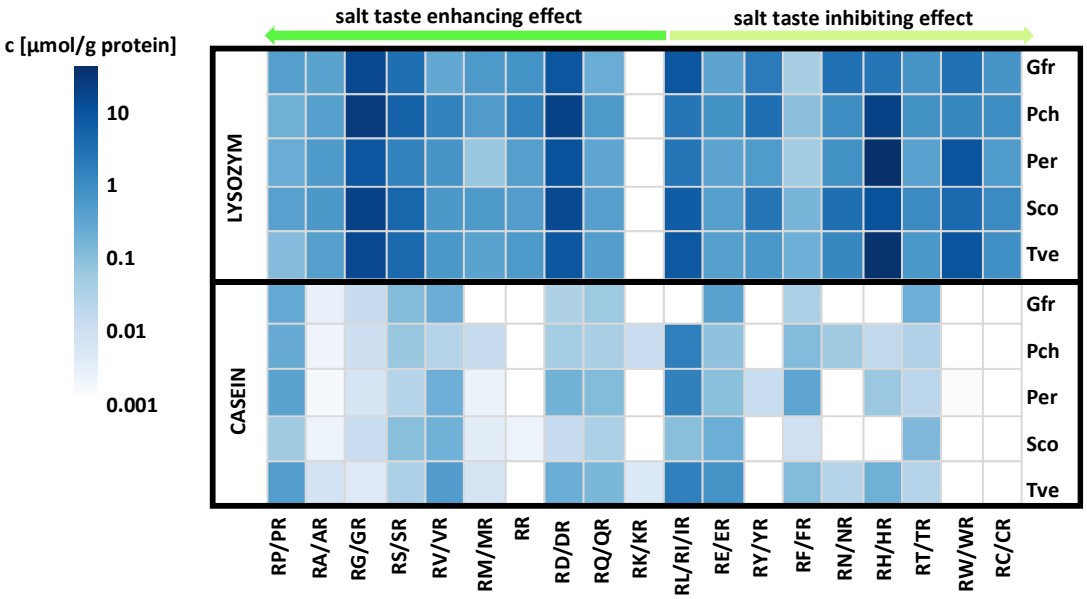
salt taste enhancing effect
salt taste inhibiting effect
c [µmol/g protein]
10
1
0.1
0.01
0.001
LYSOZYM
CASEIN
Gfr
Pch
Per
Sco
Tve
RP/PR
RA/AR
RG/GR
RS/SR
RV/VR
RM/MR
RR
RD/DR
RQ/QR
RK/KR
RL/RI/IR
RE/ER
RY/YR
RF/FR
RN/NR
RH/HR
RT/TR
RW/WR
RC/CR

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