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Hydrolysis of Wheat Gluten by Combining Peptidases of *Flammulina velutipes* and Electrodialysis

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ABSTRACT: Wheat gluten hydrolysis, used to generate seasonings, was studied using peptidases from *Flammulina velutipes* or commercial Flavourzyme. L-Amino acids were added in a range from 0.5 to 75.0 mM, and L-isoleucine, L-leucine, L-valine, and L-phenylalanine were identified as the strongest inhibitors for both enzyme mixtures. L-Serine inhibited *Flammulina velutipes* peptidases only, while L-histidine and L-glutamine inhibited Flavourzyme peptidases only. To reduce product inhibition by released L-amino acids, electrodialysis was explored. An increase of the degree of hydrolysis of up to 60% for *Flammulina velutipes* peptidases and 31% for Flavourzyme compared to that for the best control batch was observed after applying an electrodialysis unit equipped with an ultrafiltration membrane for two times 1 h during the 20 h of hydrolysis. The total transfer of free L-amino acids into the concentrate reached 25−30% per hour. Peptides passed the membrane less easily, although the nominal cutoff was 4 kDa.

KEYWORDS: *enzymatic hydrolysis, wheat gluten, product inhibition, electrodialysis, Flammulina velutipes*

INTRODUCTION

Water-insoluble wheat gluten, a byproduct of the wheat starch industry, is an abundant source of protein. Dry wheat gluten contains up to 8% moisture, varying amounts of starch, lipids, and fiber, and 80−85% proteins, which can be divided into glutenins and gliadins. Gliadins have a low level of charged L-amino acids, but are rich in L-proline and L-glutamine.[1] The full or partial hydrolysis of gluten is a universal process carried out for the production of L-glutamate-rich seasonings and bioactive peptides and for improving nutritional value, imparting texture, increasing solubility, and adding foaming or coagulation properties. The traditional hydrochloric acid hydrolysis may form toxicologically critical substances, such as 3-chloro-1,2-propanediol (MCPD) and 1,3-dichloro-2-propanol (DCP), and results in high sodium chloride concentrations in the neutralized products. The enzymatic processing alternative, introduced into industry recently, provides more sustainable and environmentally friendly conditions.[2,3]

An extensive hydrolysis, preferably using mixtures of endo- and exopeptidases, is desired to reduce the bitterness of the hydrolysate as well as to obtain a high level of free L-glutamate and small peptides.[3] Numerous studies showed the hydrolytic efficiency of commercially available peptidases, such as Alcalase, Pepsin, Neutrase, and Protamex. A maximum degree of hydrolysis (DH) of 15.8% was observed using Alcalase.[3] Other studies using trypsin and chymotrypsin achieved a DH of 2.8% and 4.3%, respectively, while Flavourzyme (from *Aspergillus oryzae*) was particularly efficient and gave 31.5%.[4,5]

The traditional heterogeneous batch fermentations last for months and are characterized by a low reaction rate.[6] It was postulated that the hydrolytic efficiency of peptidases decreases during the established processes due to accumulating soluble peptides and free L-amino acids.[7−9] Product inhibition was first described for the hydrolysis of soluble synthetic substrates.[10] Since then, the effect was recurrently observed with various peptidases and different substrates, e.g., soy protein, casein, gelatin, and wheat and corn gluten.[4,11]

Thus, high peptidase activities and long incubation times are required, reducing the economic viability of this procedure. Product inhibition could be overcome by separating the products of hydrolysis stepwise or continuously.[12] Enzyme membrane reactors (EMR) allowed a rapid separation of bioactive peptides from the hydrolysate: Cui et al.[13] used a continuous EMR with ultrafiltration (UF) membranes (cutoff 1 kDa) to produce bioactive peptides from wheat gluten. Cabrera-Padilla et al.[7] hydrolyzed whey protein using chymotrypsin and carboxypeptidase A in an EMR to obtain low levels of phenylalanine for phenylketonuria patients. They noticed that the permanent removal of L-amino acids through the membrane of the EMR implied higher reaction rates compared to that for the batch experiment.

Electrodialysis (ED) is widely applied in the food industry for water desalination, for the production of table salt or organic acids,[14−17] for deacidification of fruit juice [18] or wine stabilization,[19] but not as a separation process combined with the hydrolysis of proteins. It is an electromembrane process using an electrical potential gradient to transport ions through ion-permeable membranes from one solution to another. Commonly, ion exchange membranes are used, which are selective for anions or cations. Consequently, this technology may also separate charged L-amino acids:[20,21] Readi et al.[20] focused on the isolation of L-glutamic acid and L-aspartic acid from a mixture of L-amino acids at pH 6.0 using electrodialysis with commercially available ion exchange membranes. Because of the different pI the negatively charged Glu and Asp were

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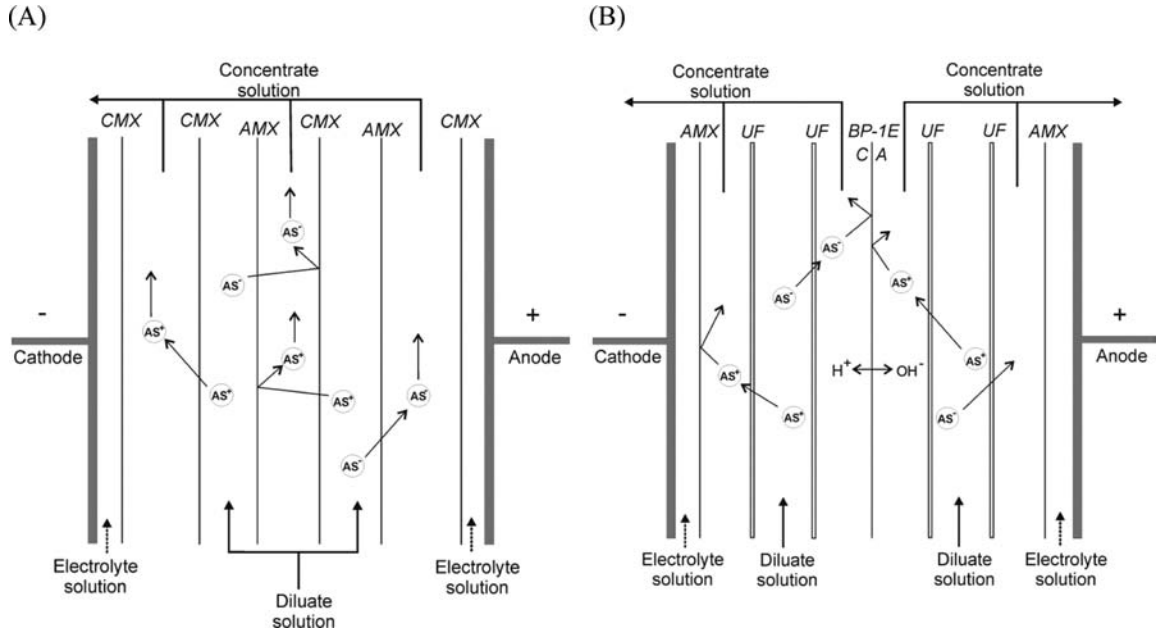

Figure 1. Schematic configuration of the electrodialysis stack for the separation of amino acids (AS), cationic membrane (CMX), anionic membrane (AMX), bipolar membrane (BP-1E) to block ion migration and delivery of the counterion, and ultrafiltration membrane (UF). (A) Traditional stack configuration with AMX and CMX membranes; (B) novel configuration with UF, BP, and AMX membranes.

separated with a recovery of around 90% and 83%, respectively. Kumar et al.[22] provided an example of the separation of L-glutamic acid (recovery 86%) and L-lysine from their mixture due to their different pI values using ion exchange membranes at pH 8. The combination of electrodialysis with ultrafiltration (UF) membranes showed potential applications for the food industry, notably for the separation and recovery of bioactive compounds from food hydrolysates[23,24] or the isolation of polyphenols from tobacco extract[25] and from green tea infusion.[26] Doyen et al.[27] demonstrated enzymatic hydrolysis of β-lactoglobulin and electrodialysis stacked with UF membranes in one step. The total migration rate of the antihypertensive peptide ALPMHIR was 66% after 240 min.

The objective of this study was to investigate the inhibitory effect of L-amino acids and peptides generated during wheat gluten hydrolysis using commercial Flavourzyme. A mixture of peptidases from the basidiomycete *Flammulina velutipes* was included into the study, because this edible fungus, when cultivated on wheat gluten, also produced savory hydrolysates.[28] Electrodialysis was configured with UF or ion exchange membranes and examined as a tool to enhance the degree of hydrolysis.

MATERIALS AND METHODS

Materials. All used chemicals and solvents were of analytical grade or HPLC grade and obtained from Sigma-Aldrich GmbH (Taufkirchen, Germany) and VWR International GmbH (Darmstadt, Germany), respectively.

Cultivation of *Flammulina velutipes*. Precultures of *Flammulina velutipes* (DSMZ, no. 1658) were grown according to Grimrath et al.[28] for seven days in 100 mL of standard nutrient medium (30 g/L D-glucose monohydrate, 3 g/L yeast extract, 4.5 g/L L-asparagine monohydrate, 0.5 g/L $MgSO_4$, 1.5 g/L KH_2PO_4, 1 mL of trace element solution, pH 6.0 [0.08 g/L $FeCl_3$, 0.09 g/L $ZnSO_4 \cdot 7H_2O$, 0.005 g/L $CuSO_4 \cdot 5H_2O$, 0.027 g/L $MnSO_4$, 0.4 g/L Titriplex III]) at 150 rpm and 24 °C. Twenty milliliters of the mycelium culture was separated by centrifugation (10 min, 5000 rpm, 4 °C) and washed two times with 20 mL of mineral salt medium (MM) (0.5 g/L $MgSO_4$, 1.5 g/L KH_2PO_4, 1 mL of trace element solution, pH 6.0). For the main cultures, 230 mL of fresh MM with 4% dry sterilized (130−135 °C, 8 h) gluten was inoculated with 20 mL of the prewashed mycelia. After 6 or 7 days, the supernatant was concentrated using ultrafiltration with a molecular weight cutoff (MWCO) of 3 kDa (Millipore, Bedford, MA) for the enzymatic conversions.

Preparation of Wheat Gluten Hydrolysates. Hydrolysis experiments were carried out with dry sterilized (130−135 °C, 8 h) wheat gluten concentrations from 1% to 10% (*w/v*) at 800 rpm, 45 °C, and pH 6.0 in 25 mM sodium acetate buffer to maintain reaction optima of the enzymes with two different peptidase mixtures from either *Flammulina velutipes* (Fve) or Flavourzyme from Novozymes (Copenhagen, Denmark), using standardized peptidase activities per milliliter of hydrolysate. The enzyme reaction was stopped after 20 h by heat inactivation at 100 °C for 10 min. For each hydrolysate, the concentration of both L-amino acids [mM/g gluten] and soluble protein [mg/L] was investigated over time. The degree of hydrolysis (DH [%]) was calculated according to Nielsen et al.[29] using eq 1

$$\mathrm{DH}\ [\%] = h/h_{\mathrm{tot}} \times 100\% \quad (1)$$

with h as the milliequivalent/g protein of α-amino group generated by the peptidase and h_{tot} as the 8.3 milliequiv/g of total α-amino groups from wheat gluten.

Quantification of the Peptidase Activity. The azocasein assay is a photometric end-point method with a substrate solution consisting of 2.5% azocasein in 0.1 M K_2HPO_4/KH_2PO_4 buffer at pH 6. Enzyme activity was measured at 37 °C in a total volume of 1.5 mL containing 200 μL of substrate and 275 μL of K_2HPO_4/KH_2PO_4 buffer (0.1 M, pH 6). Reaction was started by the addition of 25 μL of enzyme solution at 43 °C and stopped after 20 min with 1 mL of trichloroacetic acid (3%). Blanks were prepared by adding enzyme after trichloroacetic acid treatment. Blanks and samples were stored on ice for 10 min and centrifuged at 15 000 rpm for 15 min. The absorbance of the supernatant was measured at 366 nm using a spectrophotometer (UV-1650 PC, Shimadzu, Duisburg, Germany). One arbitrary U [aU] is defined as the enzyme activity that catalyzes an increase of absorbance of 0.01 at 43 °C per min.[30]

Quantification of L-Amino Acids. L-Amino acids were analyzed by RP-HPLC with fluorescence detection (Shimadzu RF-10AxL, Duisburg, Germany) and *o*PA precolumn derivatization: 110 μL of borate buffer (0.5 M, pH 10), 10 μL of hydrolysate and 20 μL of *o*PA-

Table 1. Main Characteristics of the Membranes Used

characteristics	Nadir UH004P	NEOSEPTA BP-1E	NEOSEPTA AMX	NEOSEPTA CMX
type	ultrafiltration membrane (PESH with PE/PP as backing material)	bipolar membrane composition of cationic and anionic membranes	strongly basic anion permeable	strongly acid cation permeable
electric resistance [Ω/cm^2]	–	0.9−1.7	2.4	3.0
membrane thickness [nm]	0.21−0.25	0.22	0.14	0.17
thermal stability [°C]	95	40	40	40
nominal MWCO [kDa]	4	–	0.2	0.2
company	Microdyn-Nadir	Tokuyama	Tokuyama	Tokuyama

reagent (100 mg of *o*-phthaldehyde, 1 mL of borate buffer, 9 mL of methanol, 100 μL of 3-mercaptopropanoic acid) were incubated for 2 min and stopped with 50 μL of 1 M acetic acid. Twenty microliters of the derivatized sample was injected and measured with a fluorescence detector ($\lambda_{excitation}$ = 330 nm, $\lambda_{emission}$ = 460 nm). Separation was performed with a Nucleodur C18 Pyramid column (250 × 4 mm, 5 μm, Macherey-Nagel, Düren, Germany) at a flow rate of 1 mL/min using the following gradient system with methanol (A) and 0.1 M sodium acetate containing 0.044% triethylamine at pH 6.5 (B): 0−5 min 10% A, 5−8 min 15% A, 8−40 min 60% A, 40−50 min 100% A, 50−55 min 10% A and 55−60 min 10% A. The concentration of L-amino acids was calculated according to five-point calibration curves for each L-amino acid using β-alanine (30 μM) as an internal standard. *o*PA does not react with imines; thus, L-proline and hydroxyproline and also L-cysteine were not detected.

Quantification of Soluble Protein Amount. Soluble protein concentration was measured after Lowry et al.[31] using the DC Protein assay (BIO-RAD no. 500−0111, Munich, Germany) and bovine serum albumin (0.2−1.0 mg/mL) as calibration standard.

Inhibitor Studies. Experiments to investigate the postulated product inhibition for wheat gluten hydrolysis were performed as described for the regular 20 h hydrolyses but using a total volume of 1.5 mL and taking aliquots of 60 μL.

Influence of Hydrolysate Components. Solutions of 10, 50, or 100 g/L gluten were hydrolyzed by *Flammulina velutipes* peptidases or Flavourzyme (10 kaU/mL hydrolysate) and blended with various amounts of 20 h hydrolysate solutions (0%, 10%, 30%, and 50% volume of hydrolysate/1.5 mL) into 25 mM sodium acetate buffer at pH 6. The concentrations of L-amino acids [M/h] produced were plotted against the substrate concentrations [g/L] according to Lineweaver−Burk. The influence of peptides </>3 kDa and >10 kDa on product inhibition was measured using ultrafiltered hydrolysate solutions (MWCO of 3 kDa and 10 kDa, Millipore, Bedford, MA) with 100 g/L gluten for both enzyme compositions and compared to that for a control in buffer.

Influence of Single L-Amino Acids. The inhibitory effect on *Flammulina velutipes* peptidases and Flavourzyme were determined by performing wheat gluten hydrolysis for 20 h (100 g/L gluten) in the presence of single L-amino acids, such as L-alanine, L-arginine, L-aspartic acid, L-asparagine, L-cysteine, L-glutamic acid, L-glutamine, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine (Sigma-Aldrich, Taufkirchen, Germany) in different concentrations (0.5−75.0 mM). The inhibitory effect was expressed as relative inhibition [%] of produced L-amino acids in comparison to the control without inhibitor (0% inhibition).

An enzyme activity-dependent inhibitory effect was calculated from the hydrolysis of 100 g/L gluten with different *Flammulina velutipes* and Flavourzyme activities and L-isoleucine (25 mM in the assay) as an exemplary inhibitor.

Electrodialysis. Electrodialysis experiments were carried out using the laboratory-scale EDL02 (Hescon GmbH, Engstingen, Germany) equipped with a DSE electrode to separate the L-amino acids from the wheat gluten hydrolysates during the hydrolysis. There was a diluate (feed solution = hydrolysate), concentrate (permeate solution = L-amino acids and peptides), and an electrolyte compartment (Figure 1). Different configurations of the membrane stack with anion/cation exchange (AMX/CMX) or ultrafiltration (UF) membranes are compiled in Table 1. The stack configuration with UF membranes consisted of two circuits to increase the transfer rate, wherein every diluate solution is surrounded by a concentrate solution on the anode side and a second on the cathode side. The two circuits were separated by a bipolar membrane. The function of the bipolar membrane was to block ion migration and delivery of the counterion. The effective membrane area was 57 cm^2 for each membrane, and the flow rate was 30 mL/min. Silicone/PES spacers with a thickness of 800 μm were placed between the membranes in the diluate and concentrate compartments.

Process Evaluation. The standard diluate buffer containing 25 mM sodium acetate, pH 6, and the concentrate buffer containing 2.5 mM sodium acetate, pH 6, and Na_2SO_4 were used as the electrolyte (500 mL). The experiments were carried out at constant voltage (variable from 1.0 to 12.0 V) and a maximal current density of 40 mA/cm^2. To assess the process, conductivity was measured with a SevenMulti (Mettler-Toledo GmbH, Schwerzenbach, Switzerland), and the temperature and transfer rate of the ions from the diluate into the concentrate, expressed as recovery, were determined. Some properties, such as diluate and concentrate volume, electrolyte concentration, and effective voltage were varied as outlined below. The recovery is the percentage of L-amino acids originally present in the diluate that is transported to the concentrate during 1 h on 150 mL compartment volume, as given in eq 2:

$$R\ [\%] = (n_{i,concentrate,t}/n_{i,diluate+concentrate,t}) \times 100 \quad (2)$$

with n as the amount of moles of L-amino acids present in the diluate and/or concentrate of component i [mol] at time t [h] and the recovery R.

Improvement of the Conditions of Electrodialysis. The recovery of a model solution consisting of L-glutamic acid, L-asparagine, L-serine, and L-leucine (50 mM in 25 mM sodium acetate buffer) was measured under different conditions: The compartment volume (100, 150, 200, and 250 mL) of diluate and concentrate, the pH values (5.0, 5.5, 6.0, and 6.5), the electrolyte concentration (0.25% and 2.50% Na_2SO_4), the applied voltages (1, 3, 6, 9, and 12 V), and the concentration of the L-amino acid solution (10, 50, and 100 mM) during 1 h electrodialysis were systematically varied.

Enzymatic Wheat Gluten Hydrolysis with Electrodialysis. Considering the low water solubility of wheat gluten, a discontinuous process was realized. Hydrolyses were performed with different wheat gluten concentrations in a total volume of 150 mL. Aliquots of 100 μL of diluate, concentrate, and hydrolysate were taken over time. Prehydrolysis at 45 °C (water bath, 200 rpm) and centrifugation of the prehydrolysates (5000 rpm, 5 min, 4 °C) to prepare a particle-free solution were carried out prior to electrodialysis. The separation process was performed at 12 V with a current density of 14.0 mA/cm^2, 150 mL compartment volume at 30 °C to 35 °C, and 0.25% Na_2SO_4 as electrolyte solution. Hydrolysis was performed using 10, 50, and 100 g/L gluten, *Flammulina velutipes* peptidases or Flavourzyme (1, 10, and 35 kaU/mL hydrolysate), 1, 3, or 6 h prehydrolysis in a total hydrolysis time of 20 h. Experiments were performed alternating with

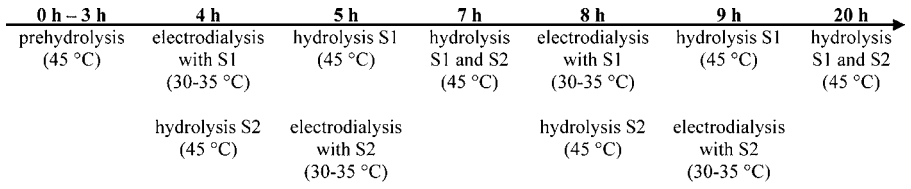

Figure 2. Experimental design of the enzymatic wheat gluten hydrolysis in combination with electrodialysis for a prehydrolysis time of 3 h, where S1 is sample 1 and S2 is sample 2.

two hydrolysis samples (sample 1 (S1) and sample 2 (S2)) as shown in Figure 2. For every experiment, a batch control was carried along. Hydrolysate samples were analyzed for peptidolytic activity and for the concentration of L-amino acids [mM/g gluten] and the soluble peptides [mg/mL].

Statistical Analysis. The inhibitory effect of L-amino acids was subjected to statistical analysis (error probability $p < 0.05$) using the one-way ANOVA of the Origin 6.1 software. All data presented are averages of duplicate measurements. Numerous experiments were conducted, and single parameters, such as enzyme activity, were changed to distinguish critical from insignificant factors; for the bulk of these measurements, the standard deviation of repetitions was typically below 5%.

RESULTS AND DISCUSSION

Inhibitory Effect of Components of Wheat Gluten Hydrolysate. To evaluate the inhibitory effect of hydrolysis products and to determine the type of inhibitor, the reaction rate at different substrate concentrations was investigated by adding various volumes of hydrolysate solutions. For each experiment, the concentration of L-amino acids produced was linearized according to Lineweaver–Burk (Figure 3). The relative inhibition increased with higher percentages of added hydrolysate fractions. Peptidases of *Flammulina velutipes* were slightly more strongly inhibited with increased DH compared to that for Flavourzyme. Peptidolysis of 10 g/L wheat gluten with 50% of hydrolysate added resulted in a reduction of L-amino acid released of up to 66% for *Flammulina velutipes* peptidases and of 60% using Flavourzyme. The inhibition for both Flavourzyme and *Flammulina velutipes* peptidases was competitive (Figure 3). Kinetic constants were not calculated, because mixtures of endo- and exopeptidases were used. A previous study on Alcalase found a competitive inhibition by the hydrolysate.[32] Apar and Ozbek[33] determined product inhibition for Alcalase acting on corn gluten hydrolysate and reported a noncompetitive inhibition type.

To evaluate which compounds were responsible for the product inhibition, wheat gluten hydrolysates were fractionated by UF. The fractions <3 kDa, 3–10 kDa, and >10 kDa were incubated in different percentages with wheat gluten and peptidase preparations. The addition of peptide fractions 3–10 kDa and >10 kDa decreased the DH compared to that for the control for both Flavourzyme and *Flammulina velutipes*. The fraction <3 kDa showed the strongest inhibitory effect on the release of L-amino acids and soluble peptides (data not shown). This fraction mainly consisted of L-amino acids and di- and tripeptides. Therefore, the focus was put on the inhibitory effect of single L-amino acids.

Inhibitory Effects of L-Amino Acids. A suspension of 100 g/L gluten was supplemented with 25 mM of each L-amino acid and incubated. The presence of single L-amino acids resulted in inhibitory effects on the release of L-amino acids as well as on soluble peptides with both Flavourzyme and *Flammulina velutipes* enzymes. Using Flavourzyme, the

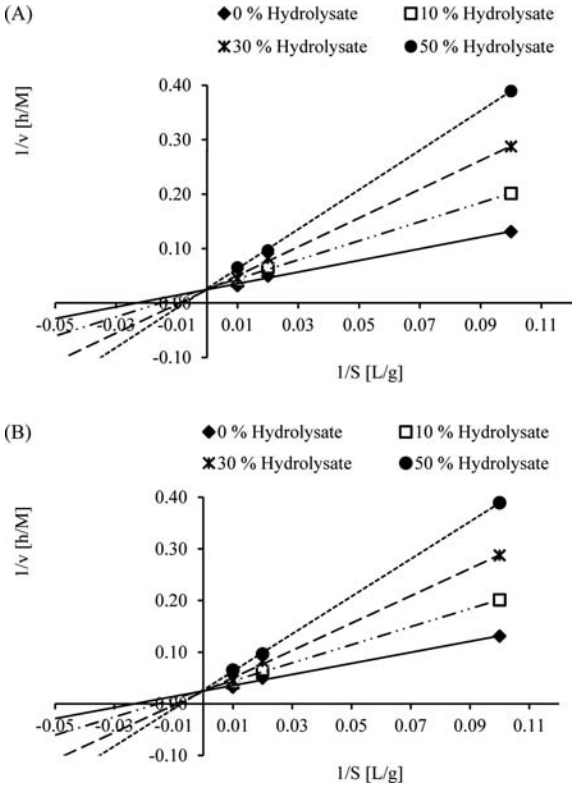

Figure 3. Lineweaver–Burk plots for the release of L-amino acids [M/h] performed with the addition of different volumes of hydrolysate and 100 g/L gluten for 20 h, at pH 6 and 45 °C with (A) Flavourzyme and (B) *Flammulina velutipes* peptidases (10 kaU/mL hydrolysate each) (0% (v/v), 10% (v/v), 30% (v/v), 50% (v/v) hydrolysate).

hydrolysis of soluble peptides was more strongly inhibited than the release of free L-amino acids (Table 2).

The hydrophobic L-amino acids L-isoleucine, L-leucine, L-valine, and L-phenylalanine were identified as the strongest inhibitors of both peptidase mixtures. Koo et al.[2] revealed a possible relationship between the DH and the hydrophobic amino acid content of wheat gluten hydrolysates obtained by Flavourzyme. The presence of 25 mM L-isoleucine resulted in a relative inhibition of >50% in comparison to the control. L-serine inhibited only *Flammulina velutipes* peptidases, while L-histidine and L-glutamine inhibited only Flavourzyme peptidases (Table 2). The inhibitory effect of L-glutamine is remarkable, because this substance is precursor to L-glutamic acid, the major target compound of the entire process.[34,35] Bacon[36] supposed that the L-amino acids remain in the active site of the peptidase, thereby preventing the attachment of a new substrate molecule and resulting in a competitive inhibition, as was found in this work (Figure 3). L-Leucine was identified as a competitive inhibitor of an intracellular aminopeptidase of *Lactobacillus acidophilus*.[37] Derivatives, such

Table 2. Inhibitory Effect [%] of L-Amino Acids [25 mM each] compared to the Control without Inhibitor added (0% inhibition) during Hydrolysis of 100 g/L Gluten for 20 h, at pH 6 and 45 °C with Flavourzyme or *Flammulina velutipes* Peptidases (10 kaU/mL each), Respectively

		Relative inhibition of the L-amino acid release [%]		Relative inhibition of the soluble peptide release [%]	
L-amino acid		Flavourzyme	*Flammulina velutipes*	Flavourzyme	*Flammulina velutipes*
alanine	Ala	–	–	23.1 ± 2.7	–
arginine	Arg	–	–	–	–
asparagine	Asn	–	–	26.4 ± 1.2	–
aspartic acid	Asp	–	–	–	–
cysteine	Cys	–	–	–	–
glutamic acid	Glu	–	–	–	–
glutamine	Gln	23.5 ± 2.0	–	48.9 ± 3.8	–
glycine	Gly	–	–	–	–
histidine	His	39.5 ± 0.8	–	48.3 ± 1.8	–
isoleucine	Ile	54.5 ± 0.8	57.1 ± 1.3	34.4 ± 4.1	51.3 ± 1.1
leucine	Leu	42.4 ± 2.8	50.2 ± 1.1	26.5 ± 4.4	22.2 ± 2.4
lysine	Lys	–	–	–	–
methionine	Met	–	–	–	–
phenylalanine	Phe	34.9 ± 4.0	21.9 ± 1.8	37.1 ± 1.7	30.2 ± 3.9
proline	Pro	–	–	–	–
serine	Ser	–	34.3 ± 1.8	–	16.7 ± 1.8
threonine	Thr	–	–	6.5 ± 0.8	–
tryptophan	Trp	–	–	–	–
tyrosine	Tyr	–	–	–	–
valine	Val	28.6 ± 0.6	51.1 ± 3.5	14.0 ± 1.0	27.7 ± 0.3

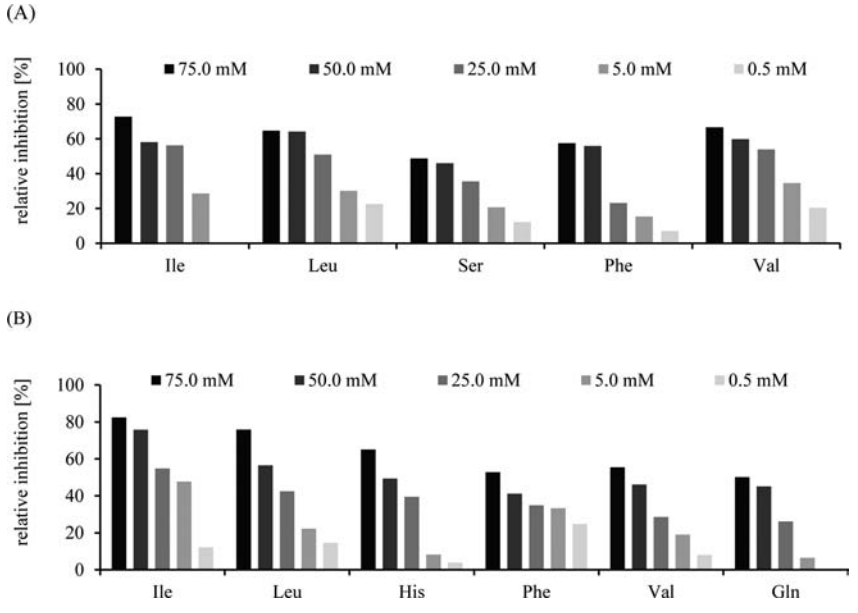

Figure 4. Concentration-dependent inhibitory effect [%] of L-amino acids [0.5, 5, 25, 50, 75 mM] during hydrolysis of 100 g/L gluten for 20 h, at pH 6 and 45 °C with *Flammulina velutipes* (A) or Flavourzyme (B) (10 kaU/mL hydrolysate each) expressed as release of L-amino acids in comparison to the control.

as isoleucine-thiazolidine were described as a dipeptidyl peptidase IV inhibitor.[38] L-Amino acids were also found to feedback inhibit in allosteric systems, such as L-isoleucine for the biosynthetic L-threonine deaminase[39] and L-histidine for the ATP-PRPP-pyro-phosphyrolase (*S. typhimurium*).[40]

The spectrum of the L-amino acids released varied depending on inhibitor and peptidase preparation. L-Leucine and L-isoleucine showed similar effects in reducing almost all L-amino acid concentrations, most likely because of their structural similarity. In the case of Flavourzyme, the incubation with L-leucine or L-isoleucine showed a decreased production of L-threonine, L-glycine, and L-valine, while *Flammulina velutipes* peptidases remained unaffected. The release of L-glutamine was reduced in the presence of every L-amino acid.

To explore critical inhibitory thresholds, the L-amino acids were added in a range from 0.5 to 75.0 mM (Figure 4). With increasing concentration of the respective L-amino acid the DH, measured as concentration of released free L-amino acids, decreased. Using Flavourzyme, the inhibitory effect of L-isoleucine was stronger than with L-leucine, followed by L-histidine > L-valine > L-phenylalanine > L-glutamine. The peptidases of *Flammulina velutipes* were inhibited by L-

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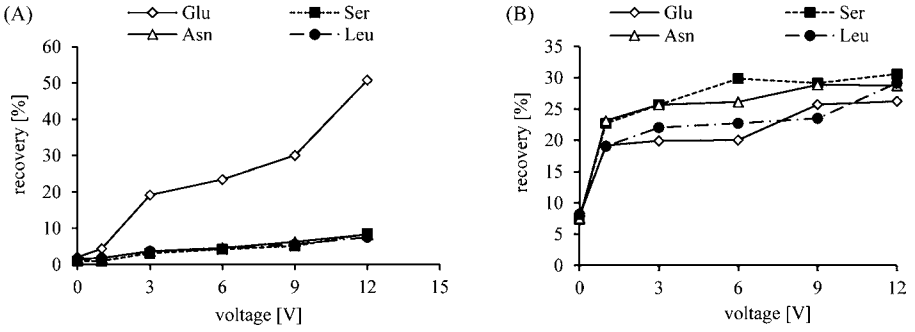

Figure 5. Effect of different voltages on the transport from diluate into concentrate per hour using (A) the AMX/CMX configuration or (B) UF membranes, expressed as recovery of the L-amino acids: L-asparagine, L-glutamic acid, L-serine, and L-leucine (50 mM model solution).

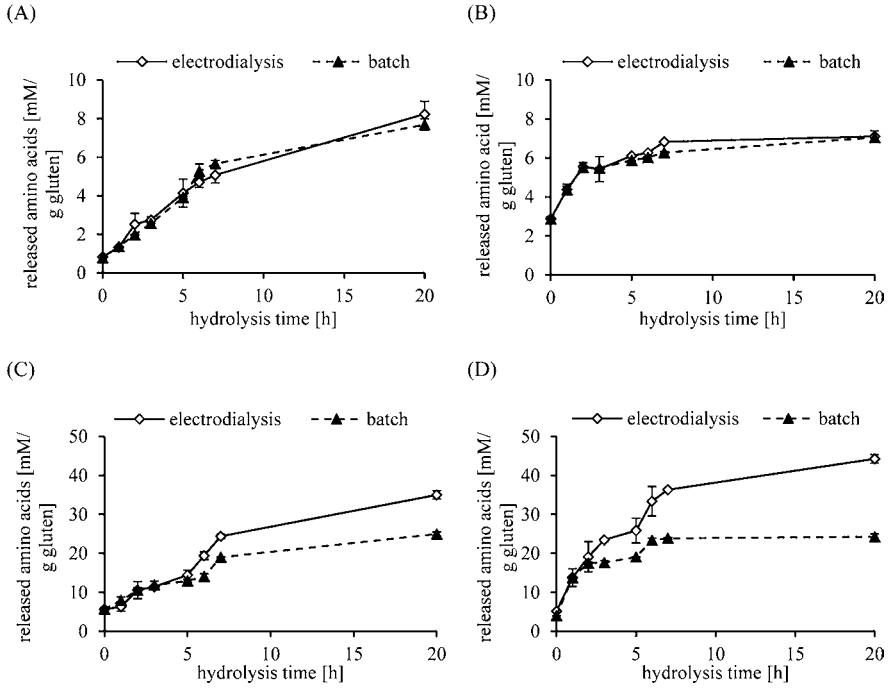

Figure 6. Wheat gluten hydrolysis in combination with electrodialysis and (A and B) the AMX/CMX configuration or (C and D) UF configuration with (A, C) Flavourzyme or (B, D) *Flammulina velutipes* peptidases (1 kaU mL^{-1} hydrolysate each) for 20 h, 10 g/L gluten at pH 6, 45 °C, 1 h prehydrolysis, 2-fold electrodialysis for 1 h at 12 V, 35 °C.

isoleucine, followed by L-valine > L-leucine > L-phenylalanine > L-serine at the same concentrations. At higher peptidase activity and L-isoleucine concentrations, the inhibitory effect on Flavourzyme was stronger than on *Flammulina velutipes* peptidases.

The following concentrations of inhibitory L-amino acids accumulated in the standard batch hydrolysis using either Flavourzyme or/the mixture from *Flammulina velutipes*: L-isoleucine (12.1 ± 1.2 mM/15.5 ± 2.3 mM), L-leucine (30.6 ± 1.1 mM/27.1 ± 2.4 mM), L-valine (16.3 ± 0.2 mM/14.0 ± 2.7 mM), L-phenylalanine (14.9 ± 0.3 mM/17.4 ± 2.0 mM), L-glutamine (67.0 ± 2.5 mM/78.1 ± 5.2 mM), L-serine (16.2 ± 0.1 mM/8.1 ± 1.7 mM), and L-histidine (6.0 ± 0.3 mM/6.5 ± 0.5 mM). L-Glutamine reached the highest concentration of all L-amino acids after peptidolysis of wheat gluten with *Flammulina velutipes* peptidases. According to Figure 4, the actual inhibitory effect of each L-amino acid in the hydrolysate control batch was estimated for Flavourzyme/*Flammulina velutipes* peptidases after 20 h: L-isoleucine (51%/40%), L-leucine (34%/47%), L-valine (21%/40%), L-phenylalanine (28%/23%), L-glutamine (42%/0%), L-histidine (11%/0%), and L-serine (0%/14%). Obviously, the accumulated concentrations showed a strong inhibition already after 4 h of hydrolysis, for example, L-isoleucine for Flavourzyme/*Flammulina velutipes* peptidases 31%/29%. Thus, the above-mentioned L-amino acids must be separated in situ to increase the DH.

Electrodialysis Using Ion Exchange and Ultrafiltration Membranes. Two different membranes (AMX/CMX and UF) were investigated for the separation of L-amino acids (Figure 1). A model solution composed of four L-amino acids, L-glutamic acid, L-asparagine, L-serine, and L-leucine was used. Best recoveries of all L-amino acids were obtained at pH 6, 150 mL for diluate and concentrate volume, and 0.25% Na_2SO_4 as the electrolyte. These optimal conditions were similar for all membrane configurations, but the individual transfer rates of the single L-amino acids from the diluate into the concentrate compartment differed. No L-amino acids were found in the electrolyte solution at any stage of the experiments.

AMX/CMX Configuration. For the pH optimum of wheat gluten hydrolysis at pH 6, the L-amino acids dissociate into

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Table 3. Efficiency of Electrodialysis compared to the Previous Batch Process expressed as the Degree of Hydrolysis [%][a]

				degree of hydrolysis [%]		
peptidases	activity [kaU mL^{-1} hydrolysate]	wheat gluten [g L^{-1}]	prehydrolysis time [h]	batch	electrodialysis	difference
Fve	1	10	1	11	27	16
Fx	1	10	1	10	17	7
Fve	35	10	1	30	48	18
Fx	35	10	1	29	38	9
Fve	1	10	3	11	17	6
Fx	1	10	3	8	10	2
Fve	10	50	3	13	20	7
Fx	10	50	3	14	18	4
Fve	10	100	6	16	22	6
Fx	10	100	6	15	17	2

[a]Wheat gluten hydrolysis for 20 h plus electrodialysis (2 × 1 h), at pH 6 and 45 °C with Flavourzyme (Fx) or *Flammulina velutipes* (Fve) peptidases, respectively.

cations (His, Arg, Lys), anions (Asp, Glu), and zwitterions (Ser, Gly, Thr, Ala, Pro, Val, Leu, Ile, Phe). Electrodialysis configured with AMX/CMX showed a low transfer of L-asparagine 8%, L-serine 8%, and L-leucine 8% per hour at pH 6. In contrast, L-glutamic acid (pI = 3.22) was transferred with a recovery of 51% at 12 V and 57 cm^2 (Figure 5 A). Zhang et al.[41] reported the separation of L-glutamic acid from an isoelectric supernatant at pH 3 with a similar recovery of 30% per hour and an effective area of 147 cm^2 at 15 V. On the basis of different pIs, charged L-amino acids, such as L-glutamic acid (pI = 3.22), can be easily separated from others. At pH 6, neutral L-amino acids, such as L-leucine (pI = 5.98), L-asparagine (pI = 5.41), and L-serine (pI = 5.68), possess a low mobility in the electric field.

UF-Membrane Configuration. For the separation of all L-amino acids, especially the hydrophobic L-leucine, L-isoleucine, L-valine, and L-phenylalanine, UF membranes (cutoff 4 kDa) were investigated which classified molecules according to charge and molecular mass. All L-amino acids were transported at similar rates. Compared to the traditional stack configuration, the UF membranes were characterized by increased ion mobility due to lower retention and membrane interaction. The recovery of the L-amino acids increased with higher voltages (Figure 5 B). Best transfer rates were achieved with 12 V: L-asparagine 25 ± 2%, L-glutamic acid 21 ± 2%, L-serine 26 ± 1%, and L-leucine 25 ± 3% per hour. Without voltage, the transfer rates varied between 5% and 8%. Due to the increased pore size and ion mobility, this osmosis effect was higher for the electrodialysis unit configured with UF (cutoff 4 kDa and four concentrate solutions) membranes than for the AMX/CMX configuration (1−2%). It is supposed that the smaller pore size of the AMX/CMX membranes (cutoff 200 Da; manufacturer's data) and only three concentrate solutions caused an increased barrier. When UF membranes were stacked in the electrodialysis cell, an increase of the applied voltage (from 1 to 12 V) resulted in a higher electrophoretic velocity [42] and an increase of the transfer rate, e.g., for L-asparagine 6%, for L-glutamic acid 7%, for L-serine 8%, and for L-leucine 10% per hour. The tested conditions did not result in a typical linear correlation with the voltage applied (Figure 5 B).[24] Using different concentrations of the model solution (10, 50, and 100 mM), no significant changes of relative recovery were observed.

Combination of Electrodialysis and Wheat Gluten Hydrolysis. When electrodialysis using AMX/CMX membranes was combined with 1 h of prehydrolysis (1 kaU/mL hydrolysate *of Flammulina velutipes* peptidases or Flavourzyme activity (Figure 2)), no major difference in the DH expressed as released amount of L-amino acids between batch and electrodialysis was obtained for either one of the peptidase preparations (Figure 6A,B). Only L-aspartic acid, L-glutamic acid, and L-lysine were effectively transported (up to 50%) after 1 h at a current density of 14.0 mA/cm^2. Product inhibition was not alleviated, because the inhibitory L-amino acids remained in the hydrolysate. The average transfer rates of free L-amino acids from diluate to concentrate achieved 7−10% for every electrodialysis period. Sandeaux et al.[21] reported a high transfer of cationic and anionic L-amino acids at pH 5 to 6 of up to 100% (88% for L-Glu) after 2 h at 30 mA/cm^2 current density from protein hydrolysates using electrodialysis with AMX/CMX membranes (Figure 6A,B). They also obtained low transfer rates of 4% for the zwitterionic L-amino acids, such as L-leucine and L-phenylalanine.

Because the objective of this study was not the selective removal of individual L-amino acids but the removal of all inhibitory compounds, other membranes were evaluated. Using UF membranes, the average transfer rates varied from 22% to 30% after 1 h at 14 mA/cm^2 current density. The conductivity was continuously measured at the beginning and at the end of each electrodialysis event. During 1 h electrodialysis (UF-membrane configuration) with 10 g/L gluten hydrolysate and 1 kaU/mL peptidase activity, the conductivity in the diluate decreased (usually from 21 to 23 mS/cm at 35 °C to 16−18 mS/cm), while it increased in the concentrate (from 0 mS/cm at 35 °C to 6−7 mS/cm) due to the transfer of L-amino acids. The conductivity of the electrolyte solution was constant during the process. The pH was measured before and after electrodialysis, and variations were detected for neither the UF configuration nor the AMX/CMX configuration, because of the buffer solution in both compartments (diluate and concentrate).

L-amino acids possessing inhibitory properties were effectively removed from the hydrolysates obtained using Flavourzyme or *Flammulina velutipes* peptidases: L-isoleucine (29%/27%), L-leucine (29%/24%), L-valine (27%/27%), L-phenylalanine (28%/23%), L-glutamine (25%/26%), L-histidine (24%/25%), and L-serine (30%/22%). As a positive side effect, soluble peptides were also transported.[27] Actual transfer rates of soluble peptides <4 kDa ranged from 7 to 10% per hour. However, after 20 h of peptidolysis, no further increase of the concentration of soluble peptides was noticeable compared with the batch experiment. This may be explained by a rapid degradation of these peptides through exopeptidases.

A declining increase of L-amino acids was found after 7 h of hydrolysis for all batch experiments (Figure 6 A–D).[2,5] Upon the partial removal of the inhibitors, the release of free L-amino acids increased compared to that for the batch control (Figure 6 C,D). Both enzyme mixtures produced similar amounts of L-amino acids in the batch process: Flavourzyme 19.3 ± 0.1 mM/g gluten, and *Flammulina velutipes* peptidases 20.3 ± 0.8 mM/g gluten after 20 h (Table 3, Figure 6 C,D). Supported by electrodialysis, both peptidases showed a higher release compared to that for the batch control: 29.4 ± 0.9 mM/g gluten for Flavourzyme and 39.1 ± 1.2 mM/g gluten for *Flammulina velutipes* (Figure 6 C,D). Several reasons may explain the different behaviors of both peptidase mixtures: Flavourzyme was inhibited by more L-amino acids compared to that of *Flammulina velutipes* peptidases. Flavourzyme inhibitors were not adequately separated and still caused a strong inhibitory effect. Other possible explanations are the different contributions of exopeptidases and endopeptidases, the loss of peptidase activity during the process, or the lack of cleavage sites in gluten. For the batch process, a decrease of the peptidase activity of about 5–10% after 20 h of peptidolysis at 45 °C was observed. The influence of the electric potential during the electrodialysis (two times for 1 h) diminished the activity of Flavourzyme by around 30% and of *Flammulina velutipes* peptidases by around 40% after 20 h. Despite the slightly lower residual activity, *Flammulina velutipes* peptidases hydrolyzed wheat gluten more effectively than Flavourzyme (Figure C,D). Further experiments with different times of prehydrolysis and substrate concentrations confirmed this and also demonstrated that the beneficial overall effects of electrodialysis were less significant for Flavourzyme than for *Flammulina velutipes* peptidases (Table 3).

Prospects of Peptidolysis Combined with Electrodialysis. Previously, enzyme membrane reactors (EMR) were used to generate bioactive peptides and to avoid the disadvantages of batch reactions, such as product inhibition or inefficient use of enzyme.[13] The significant difference and advantage of electrodialysis is the faster separation of the products of hydrolysis driven by an electric potential. Nevertheless, both processes may imply a loss of enzyme activity and membrane fouling.[43] Table 3 shows a set of experiments with different peptidase activities (1 or 35 kaU/mL hydrolysate), different substrate concentrations (1–100 g/L wheat gluten), and different times of prehydrolysis (1–6 h). The largest DH of the batch control (29% for Flavourzyme and 30% for *Flammulina velutipes* peptidases) was reached by using 10 g/L gluten and a peptidase activity of 35 kaU/mL hydrolysate. Combining electrodialysis with hydrolysis (two times 1 h), an increase of the DH using Flavourzyme of 31% and 60% using *Flammulina velutipes* peptidases was achieved. The best relative yields were obtained under the following conditions: 1 h prehydrolysis, 10 g/L gluten, and a peptidase activity of 1 kaU/mL. Improvement of 70% (from 10% up to 17% DH) for Flavourzyme and of 145% (from 11% up to 27%) for *Flammulina velutipes* peptidases were recorded (Table 3, Figure 6C,D). With longer times of prehydrolysis, higher concentrations of L-amino acids were released. Hence, the inhibitory effects started early during peptidolysis, and the electrodialysis was started too late to compensate for this (Table 3, Figure 6C,D). Similar effects were noticed with both peptidase compositions for higher wheat gluten concentrations.

With the present experimental setup, a continuous electrodialysis was not possible, because the enzyme had to be returned to the remaining insoluble substrate. So far, electrodialysis was applied two times for 1 h during 20 h (Figure 2). An improvement of the DH could be achieved by a more frequent involvement of electrodialysis in the process, an increased area of the UF membranes, or using membranes with a larger cutoff.

In conclusion, this study quantified for the first time single L-amino acids as inhibitors of Flavourzyme and of peptidases of *Flammulina velutipes* during wheat gluten hydrolysis. Hydrophobic amino acids, such as L-isoleucine, L-leucine, L-valine, and L-phenylalanine, showed a strong inhibitory effect on both peptidase mixtures. L-Glutamine, which occurs in large quantities in wheat gluten, only inhibited Flavourzyme. Preliminary observations also indicated that peptides inhibited the hydrolysis. For more detailed data, all the peptidases of the mixture should be purified and incubated with different L-amino acid or peptide concentrations. Electrodialysis configured with UF membranes (cutoff 4 kDa) was a suitable tool to reduce product inhibition. Optimized conditions of electrodialysis are expected to further improve the total release of L-amino acids.

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Notes

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ABBREVIATIONS USED

Fve, *Flammulina velutipes*; Fx, Flavourzyme; ED, electrodialysis; EMR, enzyme membrane reactor; MM, mineral salt medium; MWCO, molecular weight cut off; DH, degree of hydrolysis; UF, ultrafiltration; AMX/CMX, anion/cation exchange membranes; pI, isoelectric point

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