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A Prolyl Endopeptidase from *Flammulina velutipes* Degrades Celiac Disease-Inducing Peptides in Grain Flour Samples

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Abstract: Celiac disease (CD) is an inflammatory disorder of the small intestine. Gluten peptides are supposed to be responsible for the reaction, the best-researched of which is the so-called '33-mer'. Analogous peptides in secalins (rye) and hordeins (barley) have been described. This study presents the degradation of gliadins, glutenins, hordeins and secalins purified from the respective flours using a prolyl endopeptidase from the Basidiomycete *Flammulina velutipes* (FvpP). The flour fractions were incubated with the enzyme, and the cleavage sites were determined using high-resolution nLC-qTOF-MS/MS. For the wheat samples, eight cleavage sites in the 33-mer peptide were shown, and all of the six described epitopes were successfully cleaved. For the commercially available prolyl-specific endopeptidase from *Aspergillus niger* (An-Pep), which was used as a control, only two cleavage sites that cleaved three of the six epitopes were identified. For the secalins, four prolyl-specific cleavage sites in the CD-active peptide QPFPQPQQPIPQ were found for the FvpP but none for the An-Pep. The CD-active peptide QPFPQPEQPFPW in C-hordein was cleaved at three prolyl-specific positions by the FvpP. The study proves the usability of FvpP to degrade CD-inducing peptides in real-grain flour samples and indicates its higher effectiveness compared with An-Pep. A clinical study would be required to assess the therapeutic or preventive potential of FvpP for CD.

Keywords: Basidiomycete; *Flammulina velutipes*; prolyl endopeptidase; celiac disease; gliadin; hordein; secalin; 33-mer

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1. Introduction

Celiac disease (CD) is an autoimmune disease that affects about 1% of the worldwide population. It is induced by gluten, the storage proteins found in grains such as wheat, rye, and barley [1]. Up to now, the only possible treatment for CD is to avoid gluten completely, which severely restricts food choices for the patients.

Gluten contains both glutelins and prolamins, the latter of which are called gliadins, secalins and hordeins in wheat, rye and barley, respectively [2]. Responsible for the immune reaction are peptides in the prolamins and glutelins [3]. The best known is the so-called 33-mer from α-gliadin (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF). It is comparatively stable against peptidolysis and contains three T-cell epitopes identified in the cells of patients suffering from CD [4]. A vast number of additional peptides has been described to induce T-cell responses, some of which are only found in secalins or hordeins (e.g., [3,5,6]). The most comprehensive list, a database provided by Allergen Online, was started in 2012 and was updated in 2018 and 2022 [7]. It now contains 1041 peptides, 679 from gliadins, 90 from secalins, 77 from hordeins and 261 from glutenins [8].

As obvious from the peptide sequences, gluten is rich in proline (10–29%) and glutamine (26–53%) and thus hard to digest by the peptidases present in the human gastrointestinal system [1,9]. An option are prolyl-specific peptidases. While prolyl endopeptidases (EC 3.4.21.26) have been long known, most are incapable of cleaving long amino acid sequences or complete proteins and thus are also called prolyl oligopeptidases [10]. Due to their limitation in substrate length, they are not suitable for the degradation of gluten without pretreatment. In 2005, Edens et al. identified the first prolyl endopeptidase that hydrolyzed intact proteins. It was found in *Aspergillus niger* and accordingly named An-Pep. Although it was originally used for the debittering of protein hydrolysates [11] and the prevention of chill-haze formation in beer [11], DSM (Heerlen, Netherlands) has since commercialized the peptidase as 'Tolerase®G' for the degradation of gluten contaminations in 'gluten-free' food products [12] as it was shown to be active under conditions similar to those in the human gastrointestinal tract [13].

Our working group previously identified a prolyl endopeptidase in the Basidiomycete *Flammulina velutipes*. Comparison with An-Pep showed a sequence identity of 54% and similarity of 39%, with the low values attributed to the evolutionary drift of the younger Basidiomycetes in comparison with the Ascomycetes. The so-called FvpP was used for the hydrolysis of α-gliadin and generated cleavage results comparable to An-Pep [14].

In this work, the FvpP was used for the hydrolysis of gluten fractions isolated from wheat, rye and barley flours. The resulting cleavage sites were compared with those generated by An-Pep and evaluated for their ability to cleave CD-active peptides. As the number of peptides known is too large to compare the cleavage sites to all of them, the 33-mer from α-gliadin and the two peptides from Wahab et al. were exemplarily chosen [3,4].

2. Results

Flammulina velutipes was cultivated for seven days in the presence of 4% (*w*/*v*) gluten as a peptidase inducer. The prolyl endopeptidase was partially purified from the supernatant using ultrafiltration and size-exclusion chromatography. Afterward, SDS-PAGE in combination with LC-MS/MS after tryptic digest of the band at 57 kDa (sequence coverage: 26.2%), which corresponds to the molecular mass predicted by ExPASy [15], was used to ascertain the presence of FvpP (Figure S1a). Its peptidase activity was verified with a casein-based zymography (Figure S1b) and by using the fluorogenic substrate Z-Gly-Pro-pNA (data not shown).

The prolamin and glutelin fractions were prepared from the respective flours after defatting using the sequential extraction procedure described by Schalk et al. [16].

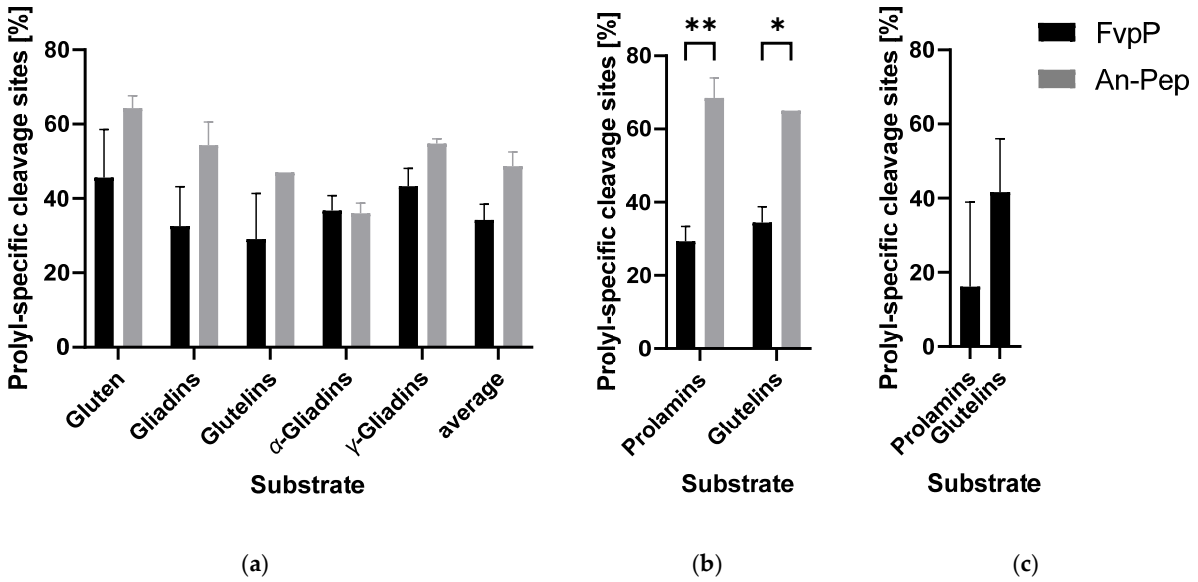
For all cleavage experiments, the commercially available prolyl-specific endopeptidase from *A. niger* (An-Pep) was used as a reference.

2.1. Wheat Samples

The prolyl-specificities of both An-Pep and FvpP were determined for all wheat substrates (Figure 1a). Apart from the samples containing α-gliadins, An-Pep showed a slightly but non-significantly higher prolyl-specificity than FvpP, with averages of 48.68 and 34.25%, respectively. Analysis of the number of prolyl-specific cleavage sites per 100 amino acids showed a slight tendency of FvpP to generate more cleavage sites than An-Pep (4.3 vs. 3.5 cleavage sites/100 AA, respectively) (Figure 2).

A total of 17 α-gliadins were identified in the different samples (Table S1), twelve in cleavage reaction setups using FvpP and five from setups containing An-Pep. All of them were aligned with the known 33-mer peptide. The alignment showed that the FvpP cleaved the 33-mer at eight different positions, while only two cleavage sites were identified for the An-Pep (Figure 3, first line). The complete multiple sequence alignments of all identified α-gliadins and the respective prolyl-specific cleavage sites are depicted in Figures S2 (FvpP) and S3 (An-Pep). The 33-mer can be subdivided into three different epitopes (A, B and C), of which A is contained once, B three times and C twice in the 33-mer

sequence. Of these six epitopes, FvpP was shown to cleave all at two separate positions, and An-Pep cleaved three of them at a single position each (Figure 3).



(**a**) (**b**) (**c**)

Figure 1. Prolyl-specificity of FvpP and An-Pep for the different flour substrates. (**a**) Wheat and (**b**) rye substrates. The cleavage specificity for the prolamins and glutelins differed significantly between FvpP and An-Pep (p = 0.0027 and 0.0253, respectively). (**c**) Barley substrates. No proteins of interest were identified in one of the glutelin samples (both wheat and rye) after cleavage with An-Pep. In the barley samples cleaved with An-Pep, no proteins of interest were identified. The remaining data results from two (An-Pep) or three (FvpP) independent cleavage experiments (n = 2 or 3). Where no significance level is indicated, the differences between the two data sets were non-significant. * $p < 0.05$. ** $p < 0.01$.

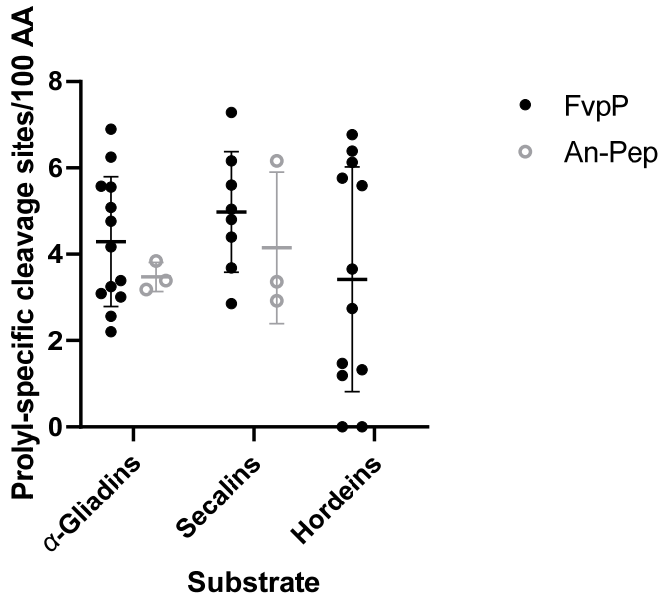


Figure 2. The number of prolyl-specific cleavage sites per 100 amino acids for α-gliadins, secalins and hordeins. Depicted is the mean with standard deviation as well as the individual values, as the number of samples differed significantly between the two endopeptidases. No hordeins were identified in the barley samples cleaved with An-Pep. The difference between the data sets was statistically non-significant.

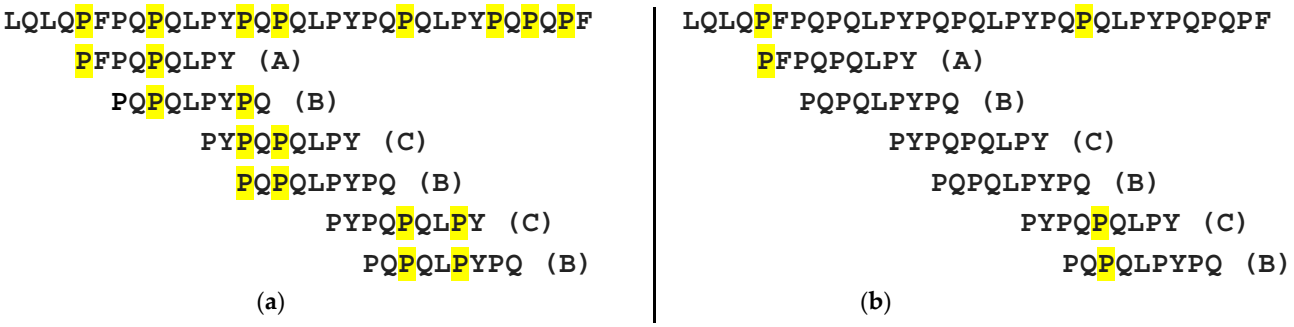


Figure 3. The 33-mer peptide from α-gliadin (first line). Cleavage sites of the FvpP (**a**) and An-Pep (**b**) are marked in yellow. Further lines depict the three T-cell epitopes (A, B and C) presumed to elicit immune reactions leading to CD and the corresponding cleavage sites.

2.2. Rye Samples

As for the wheat samples, the FvpP showed a tendency to generate more prolyl-specific cleavage sites than the An-Pep, with 5.0 and 4.1 cleavage sites per 100 AA, respectively (Figure 2). In contrast, the prolyl-specificity of the An-Pep was significantly higher (Figure 1b). For prolamins and glutelins, 68.5 and 65% of all cleavage sites were prolyl-specific. The FvpP only reached specificities of 29.3 and 34.5% for the two substrates.

In all analyzed samples, eleven secalins were identified (Table S2). Three of these were identified in samples using An-Pep for degradation. No secalins were identified in either of the two reactions with An-Pep as endopeptidase and glutelins as substrate. To check the degradation of CD-active peptides, the peptide QPFPQPQQPIPQ was chosen as an example [3]. For the best alignment, the different secalin types (ω-secalins in Figure S4, 75k γ-secalins in Figure S5, Sec1 precursors in Figure S6 and secalin precursors in Figure S7) were aligned separately with the above-mentioned peptide. While four of five potential prolyl-specific cleavage sites of the peptide were cleaved by FvpP, not a single cleavage site could be detected in any of the samples containing An-Pep (Figure 4).

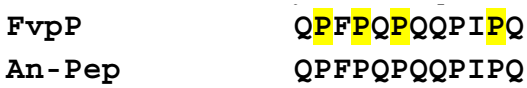


Figure 4. CD-active peptide QPFPQPQQPIPQ from secalin. Cleavage sites of the FvpP (**upper line**) and An-Pep (**bottom line**) are marked in yellow. No cleavage sites within the peptide were detected in any of the secalins identified in the An-Pep samples.

2.3. Barley Samples

In contrast to the rye samples, no secalins, glutenins or gliadins were identified in any of the barley samples. In addition, although the same number of samples was investigated for barley and rye, no hordeins were identified in any of the samples cleaved with An-Pep. Thus, a comparison between the two endopeptidases was not possible. For the samples cleaved using the FvpP, prolyl-specificities of 16.1 and 41.6% were identified for prolamins and glutelins, respectively (Figure 1c). The low prolyl-specificity for the prolamins was due to the fact that only a single B-hordein could be identified in one of the samples, and it contained only two cleavage sites, none of which were prolyl-specific. This resulted in a mathematical specificity of 0% for that sample and, thus, a high standard deviation and low average specificity. In the second prolamin sample, eight hordeins were identified, one of which contained no prolyl-specific cleavage sites either, with the others ranging between 26.2 and 40% prolyl-specificity and resulting in an average specificity of 32.3%. Likewise, the range of the prolyl-specific cleavage sites of FvpP per 100 AA varied significantly depending on the sample; with an average of 3.4 cleavages, it was also lower than for the α-gliadins and secalins.

Overall, twelve hordeins were identified (Table S3). The CD-active peptide QPFPQPE-QPFPW was only detected in the single C-hordein from the sample using prolamins as

substrate. The alignments of the B- (Figure S8) and D-hordeins (Figure S9) were therefore performed without the sequence of the peptide. While the putative γ2-hordein (hordein8) was aligned alongside C-hordein (hordein10) and the CD-active peptide (Figure S10), its sequence in the respective part of the alignment vastly differed from the sequence of the CD-active peptide. Its prolyl-specific cleavage sites that aligned with the peptide were thus not considered for the evaluation presented in Figure 5. Of the five prolyl-specific cleavage sites in the CD-active peptide, three were hydrolyzed by FvpP. One of the two non-cleaved sites was not available for cleavage as the respective proline was identified as serine in hordein10.

FvpP **QPFPQPEQPFPW**

Figure 5. CD-active peptide QPFPQPEQPFPW from C-hordein [3]. Cleavage sites of the FvpP are marked in yellow. The E and P marked in grey were identified as Q and S, respectively, in hordein10.

3. Discussion

Flammulina velutipes is an edible mushroom that is widely distributed and appreciated for its taste [17]. Its prolyl endopeptidase, FvpP, was originally purified using preparative PAGE in combination with ultrafiltration. While the resultant enzyme was clean [14], preparative PAGE is not upscalable. It was thus decided to use size-exclusion chromatography, which is based on the same separation principle and can be scaled up to industrial dimensions [18]. The resultant purity of the FvpP was significantly lower than obtained using preparative PAGE (Figure S1a), but the FvpP could be detected in both the zymography (Figure S1b) as well as via peptide fingerprinting. As additional cleavage sites would only help the degradation of gluten and toxic components were not to be expected due to the status of *Flammulina velutipes* as an edible mushroom, the partial purification was regarded as sufficient for the planned experiments.

The zymography showed some additional, larger peptidases, which were not identified (Figure S1b). These were most likely responsible for the lower specificity of FvpP in comparison to An-Pep, which was present for all samples, but especially discernable for the barley prolamins and glutelins (Figure 1). The analysis of prolyl-specific cleavage sites per 100 AA showed that these additional peptidases had no effect on the cleavage efficiency of the FvpP, as, on average, slightly more prolyl-specific cleavages sites were identified than for An-Pep (Figure 2).

For the wheat samples, the degradation of α-gliadin was evaluated in detail. The 33-mer peptide was completely degraded by the FvpP, with all possible epitopes containing two cleavage sites (Figure 3a). After treatment with An-Pep, three potential epitopes remained intact (Figure 3b), indicating a still high immunogenic potential of the treated flour fractions. Only two of the nine α-gliadins (numbers 5 and 11) identified in the sample cleaved by FvpP contained the full length of the 33-mer peptide (Figure S2), while it could be identified completely (apart from two amino acids) in all three α-gliadins identified for the An-Pep (Figure S3).

Comparable results were obtained for the secalins from the rye samples, where four cleavage sites were found for the FvpP and none for the An-Pep (Figure 4). Here, all identified secalins contained the full-length sequence of the CD-active peptide QPFPQPQQPIPQ (Figures S4–S7). In contrast to the data obtained for the 33-mer and most likely due to the shorter length of the CD-active secalin peptide, two of the eight secalins identified in the FvpP-samples showed no cleavage sites inside the CD-active peptide. This indicates that, for a safe application of the FvpP for the degradation of gluten, the activity used has to be optimized to ensure that all potential cleavage sites are, in fact, cleaved.

As no hordeins were identified in any of the An-Pep samples, no comparison between the two prolyl endopeptidases could be drawn. Like the other peptides, QPFPQPEQPFPW was efficiently cleaved by the FvpP at three positions (Figure 5).

For the nine different α-gliadins identified in the FvpP-samples, similar cleavage sites were obtained. It was thus surprising that the cleavage site in the α-gliadin presented in

the original FvpP paper vastly differed from those identified here. It is unlikely that this was due to the different preparation of the enzyme, as both enzyme preparations were active. Most likely, the differences between the substrates (pure protein vs. flour fractions) led to slight differences in protein folding and/or aggregation and, thus, vulnerability towards peptidase degradation. This is supported by the fact that in both this work and Schulz et al. [14], the cleavage sites of An-Pep and FvpP were similar.

A structural comparison between the two endopeptidases was not successful as the model of FvpP, which was obtained using SWISS-MODEL [19] with the An-Pep structure [20] as the best-fitting template, was not good. This became obvious from both its low-quality score (data not shown) as well as from the fact that the catalytic amino acids of FvpP were outside of the active center (Figure S11, [21]). While it is possible that the active amino acids were wrongly indicated by Schulz et al., this seems unlikely due to the relatively high homology of the two protein sequences near the active amino acids (Figure S12). This indicates that there are indeed relatively large structural differences between the two peptidases and also explains the differences observed in this paper. An in-depth analysis will have to wait for the structure of FvpP.

The degradation of gluten using prolyl endopeptidases as presented in this work, is only one option to deplete CD-inducing peptides. Other options include the use of germinating enzymes from barley, which efficiently ameliorated inflammatory reactions in intestinal epithelial cell models to the treated secalins. Single peptides were not evaluated [22]. Peptidases from other plants (e.g., papaya) or of bacterial or insect origin have also been utilized [23].

An alternative to the degradation of the responsible peptides was presented by Gianfrani et al., who used a microbial transglutaminase to crosslink lysine methyl esters to gliadin and CD-active peptides [24]. A study in CD patients showed that the modification was not sufficient to abolish the immune reaction in all patients but was significantly reduced [25].

Degradation studies using prolyl endopeptidases have been published analyzing the epitope degradation using T-cells (e.g., [13,26]) or the degradation of isolated peptides (e.g., [27]) or whole proteins (e.g., [22,27]), but without detailed publication of the actual cleavage sites. Stepniak et al. and Shetty et al. published cleavage sites in CD-active peptides [28,29]. Many are identical to cleavage sites identified in this study (compare Figures S2, S3 and S10), but the data is not well comparable, as not all cleavage sites are available for hydrolysis in the complete, folded proteins used in this study. Four peptides were described as cleavage products from immunopathogenic peptides in C- and D-hordeins in a different study using An-Pep. It has to be noted that the samples were first treated with An-Pep during the brewing process and later on hydrolyzed further using chymotrypsin before LC-MS/MS-analysis [30]. While the peptide in C-hordein could not be found in the single C-hordein identified in this study, the three peptides in D-hordein were marked in Figure S9. One of these peptides and its resulting cleavage sites were situated in an area where no cleavage sites could be identified in this study.

Many of the degradation studies were performed using isolated peptides, especially the 33-mer from α-gliadin [23]. The significance of the results has been questioned, as peptidase activities might differ between peptides and complete proteins or the more complex food substrates [27]. For this reason, the present work utilized grain flour fractions as substrates to analyze the degradation in a semi-complex environment. Their complexity becomes apparent when the complete multiple sequence alignments (Figures S4–S12) are considered (e.g., the α-gliadins showed significant differences even when they were isolated from the same sample).

The best solution would be the use of whole flour, but this was impossible, as the number of relevant proteins identified would have been too small for significant results. Even in the flour fractions used, only a single C-hordein could be identified and compared to the CD-active peptide.

4. Conclusions

FvpP proved capable of degrading all CD-active peptides chosen for comparison in this study at several different positions with higher efficiency than An-Pep. The results thus show that FvpP presents a promising alternative for the degradation of gluten in real food matrices. A clinical study would be required to create further evidence for the CD remedy potential of FvpP.

5. Materials and Methods

5.1. Chemicals and Reagents

All chemicals and reagents were obtained from Carl Roth (Karlsruhe, Germany), VWR (Radnor, PA, USA), Sigma Aldrich (Taufkirchen, Germany), Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland) if not stated otherwise. An-Pep was purchased from DSM (Heerlen, The Netherlands). Gluten was obtained from Nestlé (Vevey, Switzerland).

5.2. Preparation of the Flour Fractions

The prolamin and glutelin fractions were prepared from wheat, rye and barley flour exactly as described in Schalk et al. [16]. Grains of four cultivars each of wheat (Akteur, I.G. Pflanzenzucht, Munich, Germany; Julius, KWS Lochow, Bergen, Germany; Pamier, Lantmännen SW Seed, JK Bergen op Zoom, Netherlands; Tommi, Nordsaat Saatzucht, Langenstein, Germany), rye (Brasetto, Conduct, Palazzo, Visello, KWS Lochow) and barley (Grace, Marthe, Nordsaat Saatzucht; Lomerit, KWS Lochow; Sandra, I.G. Pflanzenzucht), all harvested in 2013, were mixed in a 1 + 1 + 1 + 1 mass ratio. The cultivars were selected based on their production yields and included the most commonly used cultivars in Germany in 2012/2013. The non-gluten fraction (albumins/globulins) was removed by triplicate extraction of the flour with a salt solution. The remaining sediment was extracted three times with ethanol/water (60%, *v*/*v*) to yield the prolamins, followed by triplicate extraction with propanol/salt solution (50%, *v*/*v*) under reducing conditions at 60 °C to solubilize the glutelins. All fractions were concentrated, dialyzed and freeze-dried. The dry fractions were extensively characterized using different analytical techniques [16,31].

5.3. Cultivation of Flammulina Velutipes and FvpP Purification

Flammulina velutipes (German Collection for Microorganisms and Cell Cultures, DSMZ, no. 1658) was cultivated as previously described [32]. In short, mycelium from 1 cm^2 grown agar was added to 125 mL SNL (standard nutrient liquid, 30 g/L glucose monohydrate, 3 g/L yeast extract, 4.5 g/L asparagine monohydrate, 1.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 5 µg/L $CuSO_4 \times 5\ H_2O$, 80 µg/L $FeCl_3 \times 6\ H_2O$, 30 µg/L $MnSO_4 \times H_2O$, 90 µg/L $ZnSO_4 \times 7\ H_2O$ and 400 µg/L EDTA, pH 6) and homogenized with an ULTRA-TURRAX for 30 s. The preculture was grown at 24 °C and 150 rpm for 7 days. 20 mL of the preculture were washed three times with minimal medium (MM, 10 g/L glucose monohydrate, 1 g/L yeast extract, 1.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 5 µg/L $CuSO_4 \times 5\ H_2O$, 80 µg/L $FeCl_3 \times 6\ H_2O$, 30 µg/L $MnSO_4 \times H_2O$, 90 µg/L $ZnSO_4 \times 7\ H_2O$, 400 µg/L EDTA, pH 6) and added to 250 mL MM and 10 g dry sterilized (140 °C, 14 h) gluten. After 7 days at 24 °C and 150 rpm, the biomass was separated by centrifugation (9500 rpm, 4 °C, 20 min) and discarded.

The culture supernatant was concentrated using ultrafiltration (MWCO 3000, Vivaspin 20, Sartorius, Göttingen, Germany), filtered using a 0.45 µm filter (CHROMAFIL, regenerated cellulose) and partially purified using size-exclusion chromatography. A Superdex 200 *increase column* (Bio-Rad, Hercules, CA, USA) was utilized in an NGC Chromatography System (Bio-Rad, Hercules, CA, USA) with a flow rate of 0.5 mL/min and 25 mM sodium acetate buffer, pH 5.5. The fraction containing FvpP was identified via peptidase activity assay. Active fractions were pooled and concentrated using ultrafiltration (MWCO 3000, Vivaspin 500, Sartorius, Göttingen, Germany).

For SDS-PAGE, a discontinuous gel (resolving gel: 12%) was loaded with the sample that had been heated to 96 °C for 10 min. The gel was run at 20 mA alongside a protein

standard (Precision Plus Protein Standard unstained, Bio-Rad, Hercules, CA, USA) and stained using an Imperial Protein Stain (Thermo Scientific, Waltham, MA, USA).

5.4. Peptidase Activity Determination

Prolyl-specific peptidase activity was determined using the substrate benzyloxycarbonyl-glycine-proline-para-nitroanilide (Z-Gly-Pro-pNA (Sigma-Aldrich, St. Louis, MI, USA), 2 mM in 10% DMSO) [14]. A 120 µL sodium acetate buffer, pH 5.5, was mixed with 20 µL sample and 10 µL substrate and the absorbance was measured at 405 nm for 60 min at 37 °C. Water was used for the blanks. The extinction coefficient of the produced *para*-nitroaniline was determined to be 9960 L/mol∗cm. 1 U of peptidolytic activity was defined as the concentration of enzyme required to produce 1 µM of *p*-nitroaniline per minute.

For the zymography, a semi-native discontinuous gel (12% resolving gel with no SDS) containing 1 mg/mL casein in the resolving gel was pre-run for 1 h at 40 mA and 4 °C. After loading of the non-denatured sample (loading buffer: 150 mM Tris-HCl, 20% glycerol and 25 mg/L bromphenol blue, pH 6.8) alongside a protein standard (Precision Plus Protein Standard Dual Colour), it was run at 10 mA and 4 °C. The gel was washed three times with washing solution (25 g/L Triton X100, 6.06 g/L Tris base) and incubated overnight in 0.1 M potassium phosphate buffer, pH 6. The gel was washed three times with water and stained using an Imperial Protein Stain (Thermo Scientific, Waltham, MA, USA).

5.5. Peptide Mass Fingerprinting

The FvpP protein band was excised from a semi-native gel that had been prepared and run in parallel to the zymography (without casein in the resolving gel), cut into small pieces, dried and incubated (30 min at 56 °C) with dithiothreitol (20 mM in 0.1 M NH_4HCO_3). After discarding the supernatant, gel pieces were rehydrated in iodine acetamide solution (55 mM in 0.1 M NH_4HCO_3) for 30 min at ambient temperature in the dark. The supernatant was discarded, and proteins were digested using trypsin (34 U/mL, sequencing grade; Promega, Madison, WI, USA) in 0.1 M NH_4HCO_3 (37 °C for at least 4 h). The tryptic peptides were identified by nLC-qTOF-ESI-MS/MS using the settings and machinery described below (*cf.* 5.6) for cleavage site determination.

5.6. Hydrolysis of the Flour Samples and Cleavage Site Determination

A total of 10 g/L substrate (gluten: dry sterilized for 14 h at 140 °C) and 15.3 U/mL prolyl endopeptidase (An-Pep or FvpP) in 25 mM sodium acetate buffer, pH 5.5, were incubated for 20 h at 43 °C and 330 rpm. Three independent hydrolysis experiments were set up for FvpP and two for An-Pep. Afterwards, the peptides were separated using ultrafiltration (MWCO 3000, Amicon Ultra Centrifugal Filters, Merck Millipore, Burlington, MA, USA) for 30 min at 14,000 g.

The flow-through containing oligopeptides <3000 Da was sequenced using LC-MS. Samples were injected into a nano-liquid chromatography system (EASY-nLC II, Bruker Daltronik, Bremen, Germany) equipped with a 20 mm pre-column (C18-A1 3PCS; ThermoFisher Scientific, Dreieich, Germany) and a capillary column (0.15 × 250 mm) packed with Grace MS C18 (3 µm particles, 300 Å pore; Grace Discovery Sciences (Columbia, SC, USA). Oligopeptides were eluted by a linear gradient (300 nL/min) of water and acetonitrile (each with 0.1% formic acid (*v*/*v*)) from 95% water to 95% acetonitrile within 25 min and held for 15 min. The nano-LC system was connected to a maXis impact QTOF mass spectrometer (Bruker Daltronik) equipped with a captive nanospray ion source for electrospray ionization in the positive mode. The orthogonal time-of-flight mass analyzer was calibrated prior to analysis (ESI-Low Concentration Tuning Mix, Agilent Technologies, Santa Clara, CA, USA) and operated with an average mass resolution >30,000. Collision-induced ms/ms spectra (Ar) were recorded, and peptides from m/z 200 to 2000 were evaluated using Protein Scape 3.0 software (Bruker Daltronic, Bremen, Germany). Oligopeptide sequences were identified using the Mascot search algorithm and exclusively downloaded parts (*Triticum*

aestivum, Secale cereal and Hordeum vulgare) of the SWISS-PROT protein database (date of download: 30 March 2021).

All peptides were compared to the full-length sequence of the protein they originated from, and their N- and C-termini were marked as cleavage sites. Only cleavage sites from relevant proteins (glutenins, gliadins, hordeins and secalins) were considered for the evaluation. All cleavage sites, as well as all cleavage sites after a proline per protein, were summed up (=100%), and the proline-specificity was calculated for each hydrolysis experiment. For further analyses, only the cleavage sites after proline (i.e., 'proline-specific') were evaluated.

Multiple sequence alignments were generated of closely related proteins of interest and the relevant CD-active peptides using Clustal Omega [33], and the respective prolyl-specific cleavage sites were marked by hand. The three CD-active peptides chosen for comparison with the cleavage results were chosen in advance of the cleavage analysis to prevent biased results.

5.7. Statistics

The data sets were tested for normal distribution using the Shapiro–Wilk test. All data sets but the one for the cleavage sites per 100 amino acids and An-Pep were normally distributed. For those, multiple unpaired *t*-tests (two-tailed) were performed to evaluate the significance between two specific datasets. For the cleavage sites per 100 amino acids, multiple two-tailed Mann–Whitney tests were used instead. The significance level was set to $\alpha = 0.05$ for all tests.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/catal13010158/s1, Figure S1: SDS-PAGE-Gel and native PAGE-casein-zymography of the partially purified FvpP; Figure S2: Multiple sequence alignment of all α-gliadins cleaved by the FvpP with identified prolyl-specific cleavage sites marked in yellow; Figure S3: Multiple sequence alignment of all α-gliadins cleaved by the An-Pep with identified prolyl-specific cleavage sites marked in yellow; Figure S4: Multiple sequence alignment of all ω-secalins cleaved by the An-Pep (secalins 1 and 3) or FvpP (secalins 4 and 7) with identified prolyl-specific cleavage sites marked in yellow (An-Pep) or green (FvpP); Figure S5: Multiple sequence alignment of all 75k γ-secalins cleaved by the An-Pep (secalin2) or FvpP (secalins 5 and 9) with identified prolyl-specific cleavage sites marked in yellow (An-Pep) or green (FvpP); Figure S6: Multiple sequence alignment of all Sec1 precursors cleaved by the FvpP with identified prolyl-specific cleavage sites marked in green; Figure S7: Multiple sequence alignment of all secalin precursors cleaved by the FvpP with identified prolyl-specific cleavage sites marked in green; Figure S8: Multiple sequence alignment of all B-hordeins cleaved by the FvpP with identified prolyl-specific cleavage sites marked in yellow; Figure S9: Multiple sequence alignment of all D-hordeins cleaved by the FvpP with identified prolyl-specific cleavage sites marked in yellow; Figure S10: Multiple sequence alignment of the remaining hordeins cleaved by the FvpP with identified prolyl-specific cleavage sites marked in yellow; Figure S11: Superimposed structures of the An-Pep (PDB-ID: 7WAB, blue) and the SWISS-MODEL predicted model of FvpP (black); Figure S12: Multiple sequence alignment of the An-Pep (7wab.1.A, without signaling and propeptide) and FvpP (Model_01, without signaling peptide); Table S1: gi-numbers of the α-gliadins identified in the wheat samples; Table S2: gi-numbers of the secalins identified in the rye samples; Table S3: gi-numbers of the hordeins identified in the barley samples.

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