Recombinant Fungal Aspartic Endopeptidases: Insights into Protein Hydrolysis and Combined Effect with Pepsin for Animal Feed Application

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ABSTRACT: Protein hydrolysis under acidic conditions can improve the product quality, nutrient availability, and cost efficiency, particularly when neutral or alkaline enzymes are ineffective. S ix f ung ala spartic e ndopeptidases (FAPs) w ere recombinantly expressed as active enzymes in *Komagataella phaffi*, with peak activity between 30−50 °C and pH 3.0−4.0. Despite FAP1 yielding a higher degree of hydrolysis for soy protein isolate (SPI) than FAP4, mass spectrometry analysis revealed similar cleavage preferences for the two peptidases. FAP1 and FAP4 experienced competitive product inhibition (K_i: 2.8 mg mL^{-1}, K_m: 3.2 mg mL^{-1} for FAP1 and K_i: 9.67 mg mL^{-1}, K_m: 6.58 mg mL^{-1} for FAP4). These findings suggest that K_i and K_m values, when studied in isolation, do not always predict a peptidase's hydrolytic efficacy. Among the FAPs, FAP6 notably increased soluble protein content in animal feed by ∼3-fold. FAP1, when combined with pepsin, had a positive effect on the hydrolysis of SPI. These results underscore the potential of FAPs to hydrolyze proteins—specifically, animal feed proteins—in acidic environments.

KEYWORDS: *animal feed, fungal peptidase, acidic protease, degree of hydrolysis, product inhibition, mass spectrometry*

INTRODUCTION

United Nations' projections anticipate a global population increase of approximately 2 billion people over the next 30 years, reaching ∼9.7 billion by 2050 from the current ∼8 billion.[1] This will likely result in an increased demand for food globally. The current surge in global protein demand is not only a consequence of population growth but also driven by socio-economic factors, such as increasing incomes and rapid urbanization.[2] Addressing this rising demand will most likely intensify pressure on the world's already stretched resources, posing challenges for environmental sustainability and food security.

While exploring innovative protein sources (e.g., cultured meat and insect protein) and enhancing the sustainable production of existing sources (e.g., legumes) are imperative, the significance of animal protein demand cannot be overlooked. By 2050, the global meat consumption is expected to rise by 76%.[3] Two-thirds of the total agricultural area in the EU is allocated to livestock production, and ∼75% of protein-rich animal feed is imported from South America, impacting the environment negatively.[2] It is therefore crucial to maximize nutrient absorption from existing protein sources while simultaneously exploring alternative solutions. Feed represents ∼70% of total production cost incurred during animal husbandry while amino acids and proteins are among the costliest nutrients.[4] Efficient digestion of dietary proteins is, thus, paramount. In recent years, improvement in nutrient uptake by animals in order to reduce the amount of required feed has become a focal point in poultry research.[5] The exploration of avenues to achieve improved nutrient uptake by animals has included the investigation of exogenous proteolytic enzymes being incorporated directly as supplements to animal feed or during the feed formulation process.[6]

The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) recommends "endopeptidase" as the general term for proteolytic enzymes that hydrolyze nonterminal peptide bonds.[7] Since feed proteins are eventually processed inside the gastrointestinal tract (GIT) where acidic conditions are encountered at the beginning, an endopeptidase would need to function well under acidic conditions at normal body temperatures, be able to tolerate frequently encountered chemical species, and also be able to tolerate temperature and pH fluctuations that animal feed experiences during the manufacturing process. The incorporation of exogenous peptidases has the potential to augment protein digestibility by facilitating an increased level of protein solubilization and promoting the hydrolysis of storage proteins. This, in turn, may diminish the available substrate for undesirable hindgut fermentation, resulting in improved nutrient absorption and a consequential reduction in overall costs and environmental impact.[6,8]

Aspartic endopeptidases—also referred to as acid/acidic peptidases—are proteolytic enzymes (EC 3.4.23) that show

highest activity under acidic conditions, typically between pH values of 3.0 and 4.0.[9] Aspartic endopeptidases usually have a low isoelectric point and can work between pH values of 2.0 and 6.0. The performance and stability of aspartic endopeptidases under acidic conditions makes them uniquely suitable for application as biocatalysts in industries such as animal feed, food, and beverage.[10−12] Currently, only a handful of peptidase preparations are available for use in the animal feed industry; a few notable examples include the following: RONOZYME/ProAct 360 (by DSM-Firmenich), KEMZYME (by Kemin Industries), and Axtra PRO (by Danisco Animal Nutrition). The initial applications of commercial enzymes were established within the detergent industry, which primarily deploys enzymes under alkaline conditions. Presently, "detergent peptidases" make up the bulk of the overall enzyme market. This observation could elucidate the limited commercial availability of aspartic endopeptidases.[13] Apart from being a primary source of nutrition, proteins and protein products also function as stabilizers, emulsifiers, flavoring agents, foaming agents, and gelling agents in different food systems.[14] In the food industry, aspartic endopeptidases are frequently employed to improve functional properties of proteins and also to enhance their nutritional and flavor profile. In the dairy industry, these enzymes are employed in various cheese manufacturing processes[15] while in the baking industry, they are used to modify the structure of gluten to induce textural and flavor changes in bread.[16]

Aspartic endopeptidases are most frequently isolated from fungal sources and are typically produced as zymogens[17] that require subsequent maturation. Owing to their biochemical diversity and their susceptibility to genetic manipulation, fungi represent an excellent source for the isolation and production of aspartic endopeptidases with desired properties. The ability of fungi to grow in cost-effective substrates and to produce high titers of extracellular enzymes can also simplify downstream processing.[18] The widespread commercial availability of aspartic endopeptidases, however, remains limited.[19] This is especially true for applications in the animal feed industry. Aspartic endopeptidases possess tremendous potential for applications in the animal feed industry. Recent *in vitro* studies have demonstrated that aspartic endopeptidases can increase the availability of proteinaceous substrate to animals by enhancing protein solubility.[11,20−22] Exploration of novel aspartic endopeptidases with potential industrial applications, especially in the food and feed industry, could thus expand the existing enzyme toolbox.

The aim of this study was to produce recombinant aspartic endopeptidases from a variety of fungal sources and evaluate their proteolytic potential under acidic conditions against proteins commonly encountered in the food and feed industry—such as soy protein isolate (SPI). The applicability of the FAPs was investigated by evaluating their ability to function (a) within a wide acidic pH range, (b) at usual temperatures encountered within animal GIT, (c) in combination with pepsin, (d) at usual temperatures encountered during industrial processes, and (e) within a complex environment in which all of the components are not known.

MATERIALS AND METHODS

Chemicals and Equipment. Analytical grade reagents and chemicals were purchased from either Carl Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany), unless stated otherwise. Porcine pepsin was purchased from Carl Roth (Karlsruhe, Germany). The animal feed protein matrix was prepared in-house. Maize and soybean meal were purchased from Schröder Futtermittel (Hofheim, Germany). Soy protein isolate (SPI) (~92% w/w protein content) was purchased from MP Biomedicals (Solon, OH), whereas whey protein isolate (WPI; GermanProt9000) was sourced from Sachsenmilch (Wachau, Germany). Epoch 2, manufactured by Biotek (Winooski, VT), was used for spectrophotometric analyses. Mini Gel Tank by Thermo Fisher Scientific (Dreieich, Germany) was used for gel electrophoresis. ThermoMixer C manufactured by Eppendorf (Hamburg, Germany) was used to incubate all reaction tubes. Total nitrogen content was measured using a TNM-1, manufactured by Shimadzu Corporation (Kyoto, Japan).

Gene Fragments, Plasmids, Strains, Media, and Kits. Based on variable sequence homology, the native pro-protein (zymogen) sequences of six fungal aspartic endopeptidases (FAPs) were selected on Uniprot by BLASTing the amino acid sequence of a FAP (UniProt ID: G0R8T0) from *Trichoderma reesei* QM6a, which was previously studied by our work group.[21] The shortlisted amino acid sequences of the FAPs from species of *Aspergillus*, *Talaromyces*, *and Thielavia* were back-translated and codon optimized for expression in *Komagataella phaffii* (Table 1). The synthetic gene fragments were ordered for synthesis at Twist Bioscience (San Francisco, CA). The expression vector, pBSY2S1Z, and the expression host, *K. phaffii* BG10, were procured from BISY (Graz, Austria). *Escherichia coli* DH5α was purchased from New England Biolabs (Frankfurt am Main, Germany). The Invitrogen's *Pichia* EasyComp Transformation Kit (Thermo Fisher Scientific; Dreieich, Germany) was used to prepare and transform competent *K. phaffii* BG10 cells according to the manufacturer's instructions. Restriction digestion enzymes, ligase(s), and buffer(s) were sourced from New England Biolabs (Frankfurt am Main, Germany). All media, including Luria−Bertani (LB), yeast extract peptone dextrose (YPD), buffered minimal glycerol (BMG), and buffered minimal methanol (BMM), were prepared according to the guidelines of Invitrogen's *Pichia* Expression Kit (Publication# MAN0000012). Zeocin was purchased from Invivogen (Toulouse, France). Molecular biology kits, including plasmid miniprep, DNA purification, and gel extraction, were sourced from Zymo Research Europe (Freiburg im Breisgau, Germany).

Table 1. Source Organisms, UniProt IDs, and GenBank Accession Numbers of Synthetic Gene Constructs of the Selected Fungal Aspartic Endopeptidases (FAPs)

FAP'#'	source organism	UniProt ID	GenBank accession
FAP1	*Thielavia terrestris*	G2QYS0	OR576910
FAP2	*Aspergillus fumigatus*	B0Y1 V8	OR567098
FAP3	*Aspergillus fischeri*	A1DDK1	OR567099
FAP4	*Aspergillus terreus*	A0A5M3Z588	OR576911
FAP5	*Aspergillus niger*	A0A254UAL0	OR576912
FAP6	*Talaromyces amestolkiae*	A0A364L9W4	OR576913

Construction of pBSY2S1Z—FAP'#' Plasmids and Expression of the Fungal Aspartic Endopeptidases (FAPs). Synthetic genes for the six FAPs (zymogens) were cloned in-frame with *Saccharomyces cerevisiae*'s α-mating factor secretory signal into pBSY2S1Z, under the control of the methanol-inducible AOX1 promoter, via golden gate cloning[23] using *Sap*I restriction sites. Chemically competent *E. coli* DH5α cells were transformed with the resulting expression vectors, pBSY2S1Z—FAP'#'. Isolated recombinant plasmids from single-colony transformants, selected on low salt Luria−Bertani (LB) plates supplemented with 25 μg mL^{-1} Zeocin, were validated by gene sequencing. *K. phaffii* BG10 cells were transformed according to guidelines of the Invitrogen's *Pichia* EasyComp Transformation Kit. In brief, 3 μg of linearized (by *Sac*I-HF) pBSY2S1Z—FAP'#' plasmids were used to transform chemically competent *K. phaffii* BG10 cells.

Single colonies were screened for enzyme activity after ~24 h of induction in BMMY at 30 °C following the standard method recommended by the Invitrogen's *Pichia* EasyComp Transformation

Kit. Proteolytic activity was analyzed by employing the azocasein assay.

Quantification of Protein Content. Protein content was estimated via bicinchoninic acid assay[24] using the BCA Rapid Gold kit (Thermo Fisher Scientific GmbH; Dreieich, Germany). In short, 20 μL of sample/standard was mixed thoroughly with 200 μL of freshly prepared working reagent and incubated at 30 °C for 5 min. The blank-corrected optical density at 480 nm was measured using the microtiter plate reader after 10 s of orbital shaking. Bovine serum albumin (BSA) was used as the standard protein for generating calibration curves (0−2 mg mL^{-1}).

Deglycosylation, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), and Molecular Mass Estimation. The recombinant FAPs were deglycosylated via treatment with PNGaseF (New England Biolabs GmbH, Frankfurt am Main, Germany) according to the manufacturer's instructions. SDS-PAGE was performed by a slightly modified Laemmli[25] protocol. A 4−20% gradient gel (Novex WedgeWell Tris-Glycine, Thermo Fisher Scientific GmbH; Dreieich, Germany) was used for protein separation. Broad range protein markers (10−200 kDa; Thermo Fisher Scientific GmbH, Dreieich, Germany) were used as reference proteins for molecular mass estimation. Protein bands on the gel were visualized by Coomassie staining using InstantBlue Coomassie Protein Stain (Abcam; Amsterdam, Netherlands). The theoretical MWs of mature FAP2 and FAP3 were estimated based on the annotated mature protein sequences. The theoretical MWs of the remaining FAPs were estimated based on the manual prediction of mature protein sequences via sequence homology in UniProt.

Production of Recombinant FAPs inside Shake-Flasks. The recombinant FAPs were produced using 250 mL baffled shake-flasks. Briefly, a single-colony preculture of recombinant FAP1—FAP6 was cultivated overnight at 30 °C in 10 mL of YPD medium at 180 rpm. The precultures were used to inoculate 50 mL of BMG media in 250 mL baffled shake-flasks separately. After growth at 30 °C and 180 rpm for ∼24 h, the cells were pelleted and then resuspended in 50 mL of BMM media (0.5% v/v methanol) in 250 mL baffled shake-flasks separately. The expression of FAPs was induced for ∼48 h at 30 °C and 180 rpm with 0.5% (v/v) methanol supplementation every ∼12 h. At the end of the induction phase, the culture broths were centrifuged at 5000*g* and the supernatants were filtered through 0.22 μm membranes. Aliquots were stored at −20 °C for protein content measurements, SDS-PAGE, and enzyme activity analyses. The filtered supernatants were then dialyzed against 5 mM sodium acetate buffer (pH 5.0) for ∼48 h at 4 °C with 4 buffer changes. Aliquots of the dialyzed FAPs were stored at −20 °C for further use.

Biochemical Characterization. *Determination of Amino Groups with the Ortho-Phthalaldehyde Assay.* To quantify protein hydrolysis, newly formed primary amino groups were detected after derivatization with *ortho*-phthalaldehyde (OPA), following the method described by Nielsen et al.[26] with slight modifications.[21] The OPA assay was carried out by dispensing 25 μL of appropriately diluted supernatant of the centrifuged protein hydrolysate sample and blank into a microtiter plate well, followed by the addition of 175 μL of the OPA reagent. The absorbance was measured at 340 nm using the microtiter plate reader at 30 °C after 10 s of orbital shaking and 5 min of incubation. The calibration curve was generated by employing the method mentioned above using L-serine (Carl Roth; Karlsruhe, Germany) as the standard amino acid within the calibration range of 0−4 mM.

Determination of Endopeptidase Activity Using Azocasein as a Substrate. Azocasein was used as a substrate to determine the proteolytic activity of the recombinant FAPs according to the method of Iversen and Jørgensen with slight modifications.[21] Unless specified otherwise, a substrate stock solution was prepared by dissolving azocasein in H_2O_{dd} (3% w/v). The assay was performed as follows: 200 μL of 50 mM buffer (final concentration) of required pH and 20 μL of the azocasein stock solution were mixed in a 1.5 mL microfuge tube. The above solution was equilibrated within the specified temperature range (30−90 °C) for 5 min. The hydrolysis was initiated by adding 10 μL of appropriately diluted and separately preheated (30−90 °C) FAP. The hydrolysis was carried out within the specified temperature range (30−90 °C) in a thermo mixer at 1000 rpm. The hydrolysis was terminated at various time intervals by adding 30 μL of 2 M trichloroacetic acid (TCA). For blanks, 30 μL of 2 M TCA was added prior to the addition of the enzyme under the same conditions. The spectrophotometric analysis was carried out by dispensing 150 μL of 1 M NaOH into microtiter plate wells, followed by 150 μL of supernatant of the centrifuged hydrolysates. The absorbance was measured at 450 nm using the microtiter plate reader after 10 s of orbital shaking at room temperature. One azocasein unit (ACU) of enzyme activity was defined as the increase of 1 absorbance unit per minute at 450 nm in 0.5 M NaOH under the assay conditions described above. Proteolytic activities were calculated as the averages of triplicate measurements for each experiment.

Determination of pH-Optimum, pH-Stability, Temperature Maximum, and Thermostability. Azocasein was employed as a substrate to characterize the recombinant FAPs biochemically. The pH-optimum was determined by measuring proteolytic activities after 5 min of reaction at 37 °C. Buffers (50 mM final concentration) with overlapping pH range (sodium citrate-citric acid pH 3.0−4.0; sodium acetate pH 4.0−5.5; MES pH 5.5−6.6; MOPS pH 6.6−7.5; Tris-HCl pH 7.5−8.5; glycine-HCl pH 8.5−10) were utilized to simultaneously evaluate the effect of buffer salts on proteolytic activity. Additionally, aliquots of the FAPs were incubated for 1.5 h in the buffers described above at 4 °C, and residual proteolytic activity was measured under optimum conditions. The temperature maximum was determined by measuring proteolytic activities at the optimum pH for each FAP within the temperature range of 10−90 °C after 5 min of reaction. Additionally, aliquots of the FAPs were incubated for 20 min at temperature intervals between 4 and 80 °C. The thermostability of the FAPs was evaluated by measuring their proteolytic activities under conditions of optimum pH and temperature maximum using the aforementioned heat-treated FAP aliquots. Proteolytic activities were calculated as averages of triplicate measurements for each experiment.

Effect of Ions, Solvents, Reducing Agents, and Peptidase Inhibitors on Proteolytic Activity. Azocasein was employed as a substrate to determine the effect of mono/divalent ions, solvents, reducing agents, and peptidase inhibitors at various concentrations on the proteolytic activity of the FAPs. The assays were carried out under conditions of optimum pH and temperature maximum for each FAP. Each FAP was incubated with respective test substances (final concentrations of 0.001−10 mM) for 10 min before initiating the hydrolysis. The hydrolysis was initiated by the addition of the preheated substrate at the required temperature. Proteolytic activity observed without the addition of any test substance was defined as 100% activity. Proteolytic activities were calculated as averages of triplicate measurements for each experiment.

Determination of Potential Product Inhibition by the Azocasein Assay. The endopeptidase activity assay was performed with slight modifications to study the inhibitory effect of soy protein hydrolysate (SPH) on the proteolytic activities of the FAPs. SPH was prepared by hydrolyzing 5% (w/v) suspension of the soy protein isolate (SPI) in 250 mL of MOPS buffer (50 mM, pH 7.0) with Alcalase (2% v/v) at 45 °C for ∼24 h inside glass bottles placed on hot-plates equipped with magnetic stirrers. The hydrolysis was terminated by heat inactivation at 95 °C for 10 min. Following centrifugation, the soluble peptides present in the supernatant of the hydrolysates were freeze-dried (α 1−2 LD plus, Martin Christ Gefriertrocknungsanlagen, GmbH, Osterode, Germany). Stock solution of SPH (30 mg mL^{-1}) was prepared by dissolving freeze-dried hydrolysate in H_2O_{dd}. A stock solution of azocasein (25.2 mg mL^{-1}) was prepared in H_2O_{dd}, and subsequent dilutions (0.188−12 mg mL^{-1}) were prepared in 50 mM (final concentration) reaction buffers of the desired pH. SPH stock solution was diluted in the above solution(s) to final concentrations of 0−10 mg mL^{-1}. The hydrolysis was initiated by the addition of 10 μL of the respective FAP(s) under conditions of optimum pH and temperature maximum. The reaction was terminated after 10 min by the addition of TCA (2 M, 30 μL). Absorbance of the resulting supernatant was measured at 450 nm, as described above. Proteolytic activities were calculated as averages of triplicate measurements for

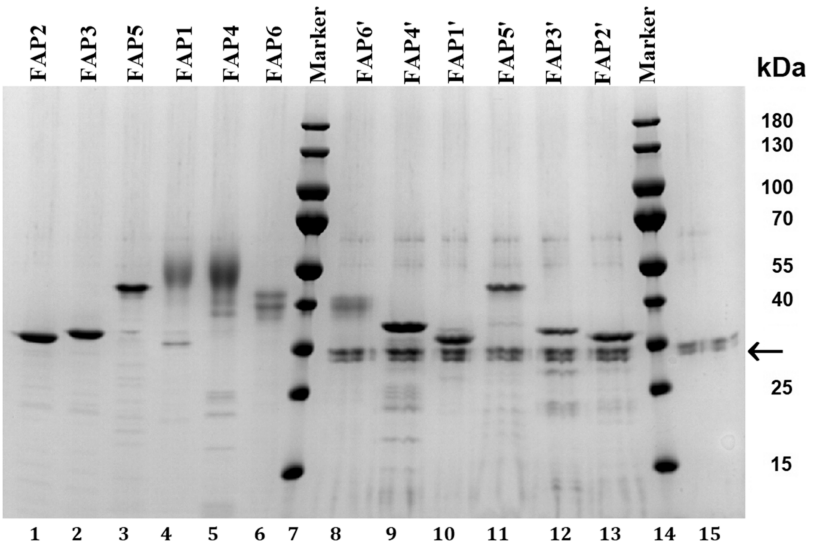

Figure 1. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis of the six FAPs after ∼48 h of induction in BMM. Lane numbers are labeled at the bottom. Lanes 1−6 contain untreated FAPs labeled at the top and lanes 8−13 contain deglycosylated FAPs also labeled at the top. Lanes 7 and 14 contain protein markers. Lane 15 contains PNGaseF indicated by "←". Protein load was ∼5 μg FAP per lane.

each experiment. The kinetic constants and the mode of inhibition were determined using the "Enzyme Kinetics Wizard" of SigmaPlot v14.5 (Systat Software Inc.; San Jose, California).

Hydrolysis of the Soy Protein Isolate. The degree of hydrolysis (DH) was analyzed by hydrolyzing the soy protein isolate (SPI) with each FAP separately. The hydrolyses were standardized at an enzyme-to-substrate ratio (E/S) of 1:700. Each FAP was applied to a 3.8% (w/v) suspension of SPI under conditions of optimum pH (50 mM buffers) and temperature maximum in a thermo mixer at 1000 rpm. The hydrolyses were terminated at multiple time intervals by dispensing 50 μL of hydrolysate into a tube containing 20 μL of trichloroacetic acid (TCA; 1 M). The blanks were run analogously with the inactivated FAPs. The DH was measured as the release of L-serine equivalents. The liberation of the free α amino groups by each FAP was determined by the OPA assay as described above.

To evaluate the difference in the DH yielded by FAP1 and FAP4, a separate set of hydrolyses was carried out by comparing individual hydrolysis of SPI by FAP1 and FAP4 to hydrolyses with sequential addition of FAP1 and FAP4 to 3.8% (w/v) SPI at an E/S of 1:33 using the method described above.

To evaluate the combined effect of FAP1 and pepsin on hyrdolysis, 3.8% (w/v) SPI was hydrolyzed by FAP1, pepsin, and FAP1 + pepsin at an E/S of 1:33 using the method described above. The hydrolysis was tracked over the course of ∼1.5 h which is the usual transit time of feed in poultry—starting at ingestion to the time feed exits the gizzard.[27] DH was calculated as averages of triplicate measurements for each experiment.

Animal Feed Hydrolysis. A representative animal feed matrix was prepared by combining maize and soybean meal in a ratio of 7:3.[28] Briefly, 70% (w/w) maize and 30% (w/w) soybean meal were mixed and then milled using a 1 mm mesh. The moisture content of the animal feed matrix was determined with a dry mass analyzer (Kern DBS, Kern & Sohn, GmbH, Balingen, Germany). Suspensions of animal feed matrix were prepared by suspending 0.2 $g_{dry\ mass}$ animal feed matrix in sodium acetate buffer (50 mM final concentration, pH 3.6) in separate 5 mL microfuge tubes. Each FAP was added to the substrate suspension at an enzyme-to-soy protein ratio of 1:700. The final volume in each tube was 5.062 mL with the reaction buffer. The hydrolyses were carried out for 90 min at 41 °C and pH 3.6 to mimic the digestive tract of chickens.[29] The enzymatic hydrolyses were terminated at time intervals of 30, 60, and 90 min by withdrawing 200 μL of hydrolysate and blank and heat inactivating it at 95 °C for 10 min in 1800 μL of H_2O_{dd}. The blanks were prepared by inactivating each FAP at 95 °C for 10 min before addition. The feed hydrolyses for each FAP were carried out as triplicate measurements. The proteinaceous nitrogen content in the hydrolysates was determined by a total nitrogen analyzer. Centrifuged samples were filtered and diluted further as needed before measurement. Nitrogen oxide was detected by chemiluminescence at 720 °C using synthetic air as carrier gas at a flow rate of 500 mL min^{-1}. The analyzer measured the samples up to five times and determined whether the standard deviation was within the specified tolerance, also ensuring that the values fit within the calibration curve (0−10 and 10−100 mg L^{-1}). The concentration of nitrogen measured by the chemiluminescence detector was recorded as a function of time. The resulting integral was considered to be a measure of the nitrogen present in the samples. The solubilized protein content of the samples was calculated by multiplying the determined nitrogen content by a nitrogen-to protein conversion factor of 6.25.[30]

Electrospray-Ionization Quadrupole Time-of-flight Mass (ESI-Q-ToF) Coupled to Ultraperformance Liquid Chromatography (UPLC). Peptides in soy protein isolate (SPI) hydrolysates generated by FAP1 and FAP4 were separated by UHPLC (Exion AD, Shimadzu; Duisburg, Germany) using a LunaOmega C-18 Polar, 1.6 μm, 2.1 mm × 100 mm column (Phenomenex; Aschaffenburg, Germany). A total runtime of 30 min was applied; starting at 90% (v/v) of eluent B (water +0.1% H_3COOH) and 10% (v/v) of eluent A (acetonitrile +0.1% H_3COOH) for 1 min, followed by a linear gradient to 90% (v/v) of eluent A over 20 min, which was held for 3 min and switched back to the initial conditions to equilibrate the column for the next run. The flow rate was constant at 300 μL min^{-1} and all analyses were carried out using a sample volume of 5 μL. The described UPLC-system was coupled directly to the interface of an X500R high resolution ESI-Q-ToF mass spectrometer (Sciex; Toronto, Canada) equipped with an electrospray-ionization (ESI) source operating in positive ion mode. The ion source was heated up to 450 °C and an ionspray voltage of 5200 V was applied. MS-experiments were carried out at a collision energy (CE) of 10 V and a declustering potential (DP) of 20 V. The acquisition range was 200 to 2000 m/z. In all experiments, nitrogen gas 5.0 was used as nebulizer, curtain, and collision gas. The resulting chromatograms were evaluated with SCIEX OS 2.2.0.5738 instrument software. For data analysis, peptides were identified using ProteinPilot Software 5.0.2 (Sciex; Toronto, Canada). The significance level for the protein threshold was set at 0.05, and the competitor error margin was 2.0. The specific parameters for the ProteinPilot Paragon method included setting the sample type to identification, not applying cysteine alkylation or digestion, specifying the species as Gylcine Max, using

the Uniprot-all-OriginalCopy.fasta database, and setting the search effort to thorough ID.

The amino acid sequences of the identified peptides were subsequently analyzed to assess the occurrence of terminal and penultimate amino acids and to shine light on the cleavage preferences of FAP1 and FAP4.

RESULTS

Production of Recombinant FAPs in *K. phaffi* and Their Subsequent Biochemical Characterization. The FAPs were expressed as preproproteins using the native pro-protein sequences (where applicable) and *S. cerevisiae*'s α-mating factor secretory signal. Each FAP was secreted into a BMM culture broth as an active (mature) enzyme. No significant increase in the concentrations of FAPs was observed on SDS-PAGE after ∼44 h of induction. Since low molecular weight impurities were removed during dialysis (Figure S1) and FAPs migrated as singular bands, further purification via column chromatography was not performed. FAP1, FAP4, and FAP6 appeared glycosylated on SDS-PAGE. All of the FAPs were analyzed again on a gradient gel under reducing conditions after deglycosylation (Figure 1). FAP6 still retained some glycosylation which could indicate the presence of a glycosidic linkage other than N-linked glycosylation. The MWs alongside protein content and specific proteolytic activity of each mature FAP after dialysis are presented in Table 2. In a decreasing order of magnitude, FAP3, FAP1, and FAP2 yielded the highest specific azocaseinolytic activities while FAP5, FAP6, and FAP4 yielded the lowest azocaseinolytic activities.

Table 2. Summary of Production Parameters of Each FAP Including MW, Protein Concentration, and Specific Azocaseinolytic Activity

parameter	FAP1	FAP2	FAP3	FAP4	FAP5	FAP6
apparent glycosylation	yes	no	no	yes	no	yes
approximate MW, apparent [kDa]	36	32	33	37	43	40
protein concentration, predialysis [mg mL^{-1}]	0.75	0.82	0.67	0.88	0.68	0.82
protein concentration, postdialysis [mg mL^{-1}]	0.2	0.28	0.16	0.52	0.32	0.42
specific azocaseinolytic activity, postdialysis [ACU mg^{-1}]	636	450	789	34	212	54

The proteolytic activity of all six FAPs under various pH and temperature conditions as well as in the presence of chaotropes, organic solvents, and different divalent cations was evaluated using azocasein as a substrate. Buffers with an overlapping pH range were used to analyze the effect of buffer salts on proteolytic activity. All six FAPs had a pH optimum between pH 3.0 and 4.0, while maintaining up to 40% activity until pH 5.5. FAP1−FAP5 lost between 70 and 90% of proteolytic activity below pH 3.0, whereas FAP6 notably retained ∼60% of its proteolytic activity at pH 2.5. The FAPs exhibited maximum activity within the strict temperature range of 40−60 °C.Figures 2 and 3 illustrate the exact pH preference and temperature profile of each individual FAP.

The temperature and pH stability of each FAP were also evaluated. The exact effect of various temperatures and pH conditions on the stability of the FAPs is summarized in Figures S2 and S3. Apart from the notable exceptions of FAP1 and FAP5, each FAP tolerated temperatures up to 60 °C without losing more than 40% of proteolytic activity. Except FAP4 and FAP5, none of the FAPs lost any significant proteolytic activity during 1.5 h of incubation between pH 2.5 and 8.5.

The FAPs were confirmed to be aspartic endopeptidases via complete inactivation with 0.001 mM Pepstatin A. The influence of different divalent cations, solvents, and denaturing agents on the proteolytic activity of each FAP was studied to characterize its performance under variable application conditions. DMF slightly enhanced the activity of FAP3 (up to 129%) and FAP6 (up to 123%) and DMSO had no significant effect on FAP3 (100%) and FAP6 (108%), while the other solvents had a 20−60% inhibitory effect on all of the FAP, including FAP3 and FAP6. Apart from copper, which reduced enzymatic activity down to ∼50% at 1 mM concentration and down to ∼80% at 10 mM concentration, the tested metal ions had no effect on the FAPs at a 1 mM concentration. Magnesium, potassium, and calcium ions were tolerated well by the FAPs up to a 10 mM concentration. FAP3 demonstrated an increase in proteolytic activity (up to145%) in the presence of 10 mM cobalt ions, while all of the remaining FAPs, except FAP2, underwent inhibition to varying degrees (20−50% reduction). Zinc ions at 10 mM significantly inhibited FAP2, FAP3, and FAP6. FAP5 and FAP6 demonstrated an increase in proteolytic activity (178 and 130%, respectively) in the presence of 10 mM manganese ions whereas FAP2 as well as FAP4 experienced a decrease in proteolytic activity (68 and 28%, respectively). Chaotropic and reducing agents like urea, DTT, EDTA, and β-mercaptoethanol exerted no effect of the proteolytic activities of the FAPs up to a concentration of 10 mM. The results are summarized in Table S1.

Potential Product Inhibition of the FAPs. The FAPs were analyzed for product inhibition by employing azocasein as a substrate. The K_m and K_i values calculated for the six FAPs are summarized in Table 3. Most notably, FAP4 and FAP6 had the lowest substrate affinity to the synthetic substrate azocasein and also appeared to experience the least amount of product inhibition by soy protein hydrolysates (SPH). FAP1, FAP2, FAP3, and FAP5 experienced comparably higher levels of potential product inhibition with SPH while also exhibiting higher affinity toward azocasein.

Hydrolysis of Soy Protein Isolate (SPI) and Cleavage Preferences of FAP1 and FAP4. The soy protein isolate (SPI)'s degree of hydrolysis (DH) was monitored by tracking the generation of free α amino groups as determined by the OPA assay. The observed efficiency (measured as liberated L-serine equivalence) of SPI hydrolysis by the FAPs was as follows: FAP1 (∼25 mM) > FAP3 (∼24 mM) > FAP2 (∼21 mM) > FAP6 (∼20 mM) > FAP5 (∼15 mM) > FAP4 (9 mM). The SPI hydrolysis revealed different kinetics between the FAPs (Figure 4). FAP1 yielded the highest DH while still generating a steady and continuous increase in α amino groups until the hydrolysis was terminated at 460 min, whereas FAP2 and FAP6 appeared to have already yielded their individual highest DH by ∼300 min. FAP2, FAP3, and FAP6 demonstrated similar kinetics, whereas FAP4 and FAP5 performed comparably poorly. FAP4 and FAP6 had similar specific azocaseinolytic activities (34 and 54 ACU mg^{-1}, respectively). Even though FAP4's $K_m < K_i$ whereas FAP6's $K_m > K_i$, FAP6 outperformed FAP4 during SPI hydrolysis. FAP1 and FAP4, having yielded the highest and lowest DH respectively, were selected for further investigation to explore the reasons behind their differing hydrolytic performance

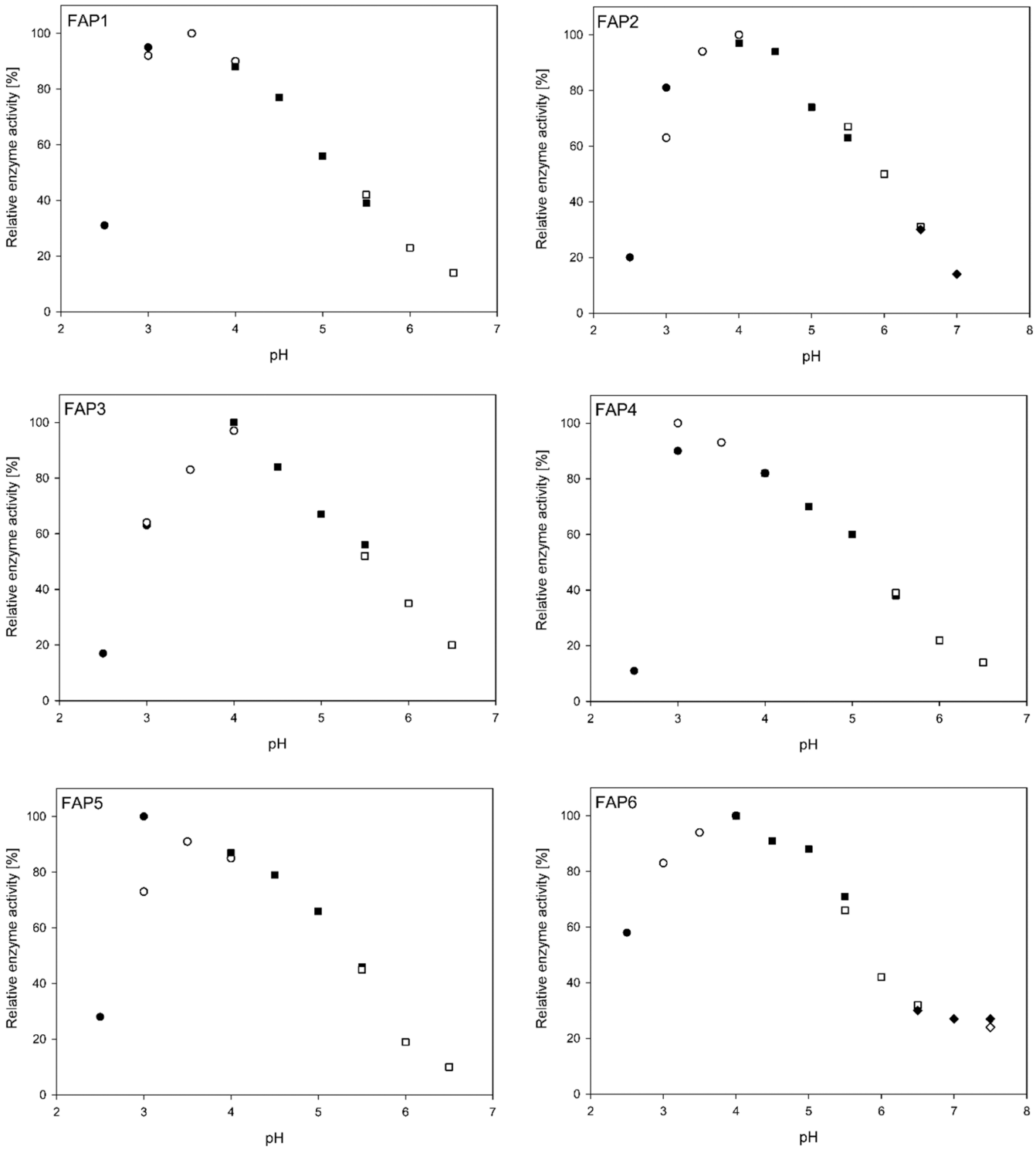

Figure 2. pH-optimum of the six FAPs using different buffers with overlapping pH range. Buffers (50 mM): "●" Glycine-HCl; "○" Sodium citrate-citric acid; "■" Sodium acetate, "□" MES, "♦" MOPS, "◊" Tris-HCl. The reported values are averages of triplicate measurements.

besides the difference in their specific proteolytic activities against the synthetic substrate "azocasein." Sequential hydrolysis of SPI at a higher E/S ratio (1:33) by FAP1, followed by FAP4 and vice versa showed that the DH increased after the sequential addition of FAP1 but did not change at all after the sequential addition of FAP4 (Figure 4). Additionally, applying a higher dosage of FAP4 did not increase its hydrolytic performance. The final DH yielded by FAP4, even when applied at a 21-times higher dosage, remained at a comparable level (~10 mM L-serine liberation at E/S = 1:700 vs ~9 mM L-serine liberation at E/S = 1:33 over the course of ~7 h). The hydrolysates yielded at 600 min by FAP1 and FAP4 individually were analyzed by ESI-Q-ToF to identify the peptides present inside. In total, 359 and 235 peptides were identified with ≥95% confidence level in SPI hydrolysates generated by FAPI and FAP4, respectively. In FAP1 hydrolysates, 218 unique peptides were identified, while FAP4 hydrolysates contained 94 unique peptides. The remaining 141

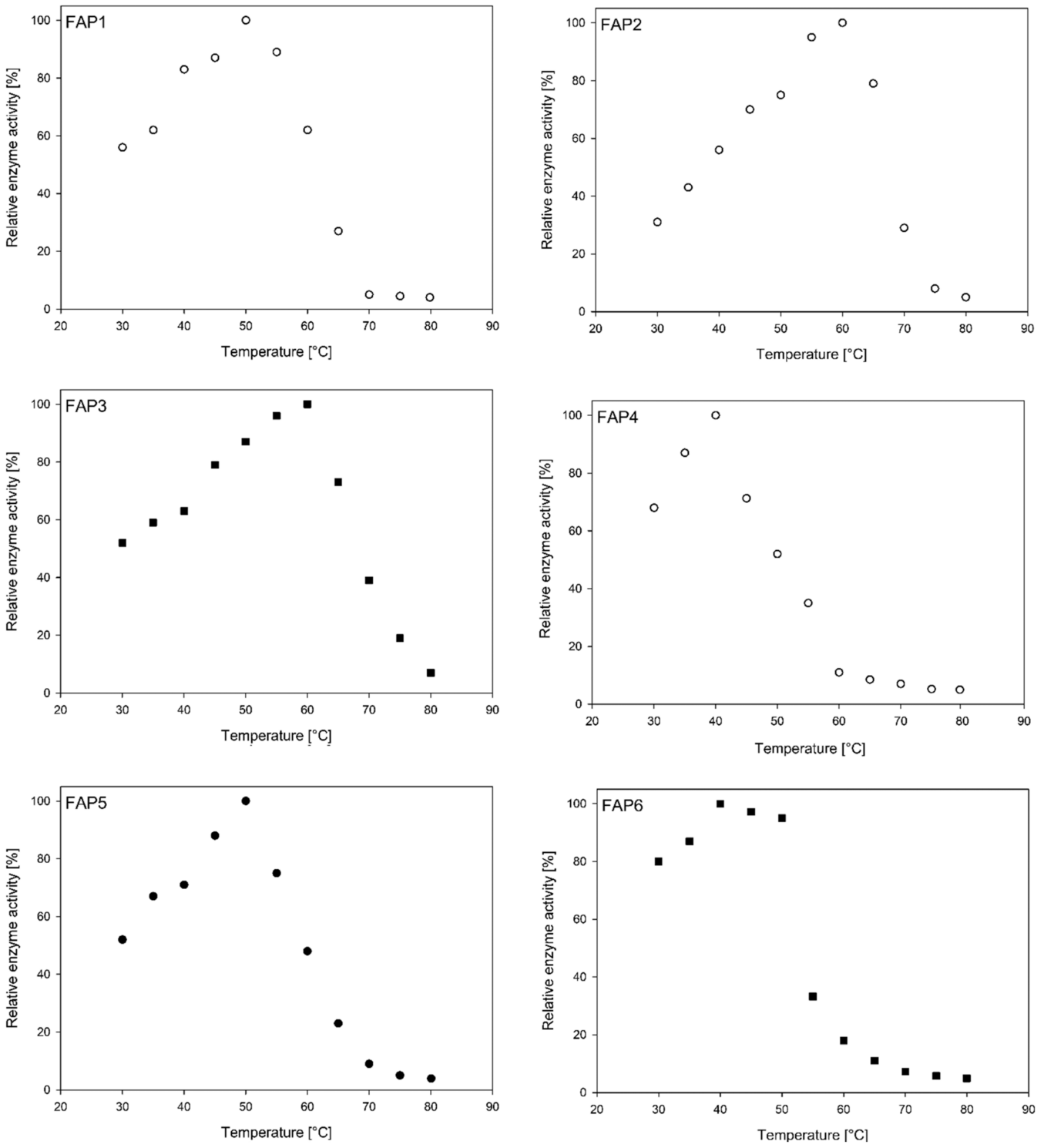

Figure 3. Temperature maximum of the six FAPs at respective pH optimum. Buffers (50 mM): "●" Glycine-HCl; "○" Sodium citrate-citric acid; "■" Sodium acetate. The reported values are averages of triplicate measurements.

Table 3. Substrate Affinity Constant "K_m" and Inhibition Constant "K_i" Determined for Each FAP Using Azocasein as a Substrate and $SPH_{Alcalase}$ as an Inhibitor at Various Concentrations

constants [mg mL^{-1}]	FAP1	FAP2	FAP3	FAP4	FAP5	FAP6
K_m (azocasein)	3.20	1.89	2.31	6.58	2.80	9.13
K_i (SPH)	2.80	3.11	2.26	9.67	2.27	5.58

peptides were identified in hydrolysates generated by both FAP1 and FAP4. These findings appeared to be consistent with the fact that the initial rate of L-serine liberation during the hydrolysis of prehydrolyzed SPI and native SPI by FAP1 remained virtually unchanged. Peptide sequences were analyzed to determine the length of peptides and the incidence of amino acids at N and C terminals to evaluate cleavage preferences of the two FAPs. Table 4 summarizes the occurrence of each amino acid found at the N and C terminals

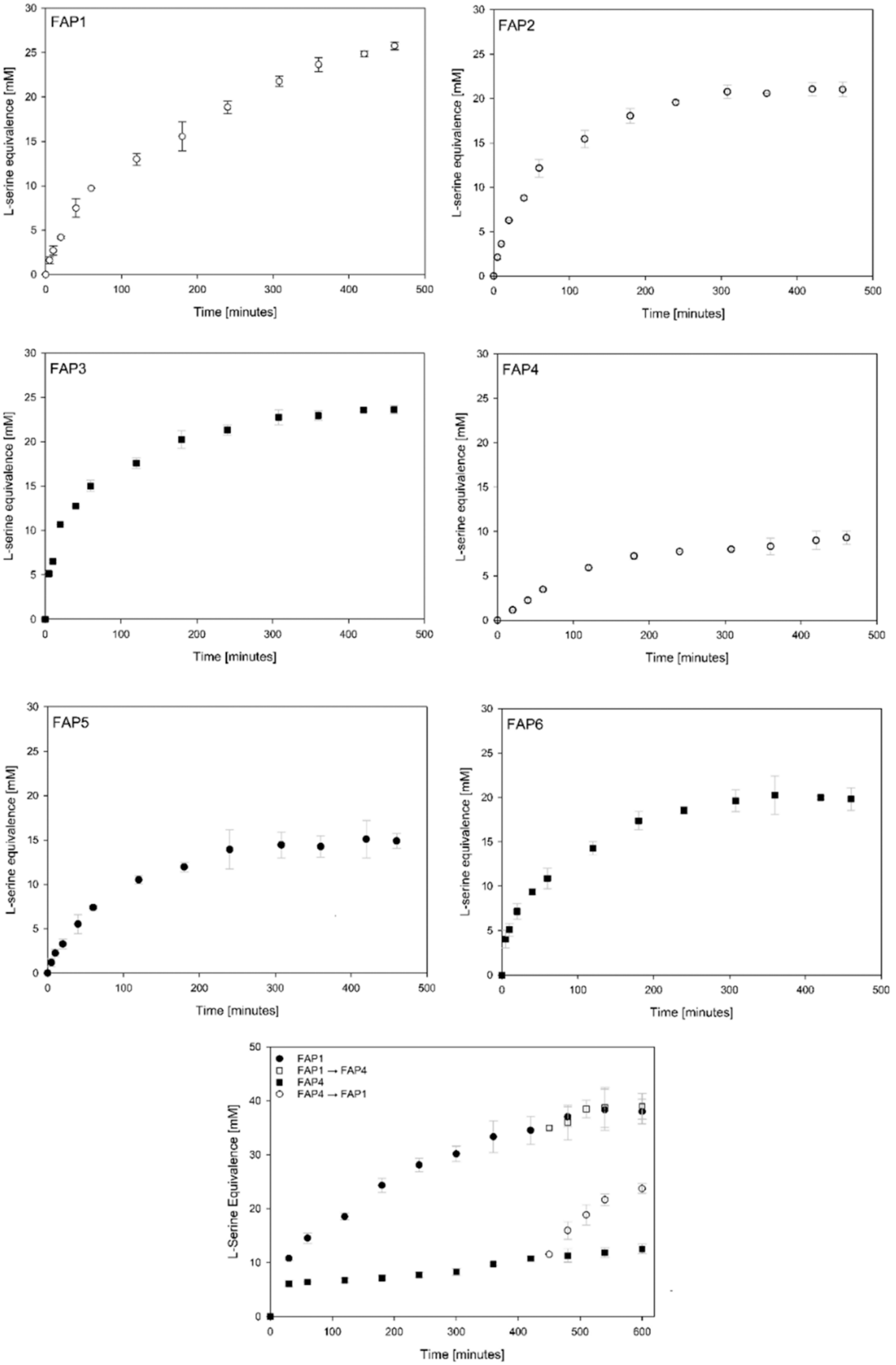

Figure 4. Liberation of L-serine equivalents during the hydrolysis of SPI (3.8% w/v) by the individual FAPs at an E/S of 1:700 and during the sequential hydrolysis by FAP1 and FAP4 at an E/S of 1:33. The reported values are the averages of triplicate measurements.

Table 4. Incidence of Terminal Amino Acids Found in the Peptides Identified with ≥95% Confidence in the SPI Hydrolysates Generated by FAP1 and FAP4 after 10 h

FAP1			FAP4	
359 peptides average length of peptides: ~10 AA			235 peptides average length of peptides: ~10 AA	
incidence of AA at N terminal [%]	incidence of AA at C terminal [%]	amino acids (AA)	incidence of AA at N terminal [%]	incidence of AA at C terminal [%]
7.2	3.6	A	3.8	3.4
0.6	13.6	R	0.4	15.3
9.2	2.8	N	10.6	3.0
7.8	3.9	D	7.7	5.5
0.0	0.0	C	0.0	0.4
11.1	7.8	E	11.5	7.7
7.2	4.5	Q	6.8	8.5
1.1	1.7	G	3.0	5.1
0.8	1.4	H	0.4	2.1
7.2	0.8	I	7.2	0.9
10.0	18.7	L	9.8	14.0
2.5	8.9	K	1.7	8.5
0.3	2.2	M	0.4	0.9
3.3	8.9	F	6.4	9.8
2.8	1.1	P	2.1	1.3
8.9	3.3	S	8.1	0.9
6.1	3.3	T	7.2	3.4
1.9	3.6	W	2.6	2.1
1.7	8.6	Y	3.0	7.2
10.0	1.1	V	7.2	0.0

of the identified peptides relative to the total number of peptide sequences identified in the hydrolysates generated by FAP1 and FAP4. The average length of 141 peptides identified in both FAP1 and FAP4 hydrolysates was ~10 amino acids. The average length of 218 unique peptides generated by FAP1 was ~11, whereas the average length of 94 unique peptides generated by FAP4 was ~9. Neither of the FAPs showed any preferential bias toward polar (charged and uncharged) or nonpolar amino acids. Therefore, it was concluded that FAP1 and FAP4 hydrolyze SPI nonspecifically since no preferred cleavage sites could be identified with the experiment. A further analysis of identified amino acids at the N and C terminals can be found in Table S2. The complete list of identified peptides can be found in Table S3.

In our study, the application of the recombinant FAPs to hydrolyze protein in a sample of a representative animal feed matrix (AFM) was tested in an *in vitro* assay. The hydrolysis by each FAP was carried out at 41 °C and pH 3.6 to mimic the digestive tract of chickens. The results are summarized in Figure 5. Since animal feed forms a viscous suspension whose homogeneity is difficult to control, the resulting data was also normalized to depict the fold-increase in soluble protein content in order to present a more accurate comparison of the FAPs' hydrolytic potentials. After 30 min of hydrolysis, the soluble protein content of the animal feed suspension increased by up to 109%. At the end of the hydrolysis (2 h), an increase of >200% was observed in the relative soluble protein content compared to the reference. The observed efficiency of animal feed hydrolysis by the FAPs (measured as fold-increase in soluble protein content) was as follows: FAP6 (~3-fold) > FAP1(~2.7-fold) > FAP2 (~2.6-fold) > FAP3 (~2.3-fold) > FAP5 (~2.2-fold) > FAP4 (~1.8-fold). These findings are in line with the results of SPI hydrolysis by the FAPs.

Combined Effect of FAP1 and Pepsin on Soy Protein Isolate (SPI) Hydrolysis. Individually, both FAP1 and pepsin hydrolyzed SPI to a similar degree of hydrolysis (DH; ~9 and 10 mM L-serine liberation over the course of ~1.5 h, respectively). The DH of SPI by the combined action of FAP1 and pepsin increased ~45% (Figure 6) indicating that SPI hydrolysis could benefit from the addition of FAP1 when gastric pepsin is already present.

DISCUSSION

Fungi are an abundant source of aspartic endopeptidases. Isolation and heterologous production of FAPs has been reported from species of *Saccharomyces*, *Aspergillus*, *Mucor*, *Rhizomucor*, *Candida*, and *Botrytis*.[9] Among fungi that are utilized frequently by the enzyme industry, species of

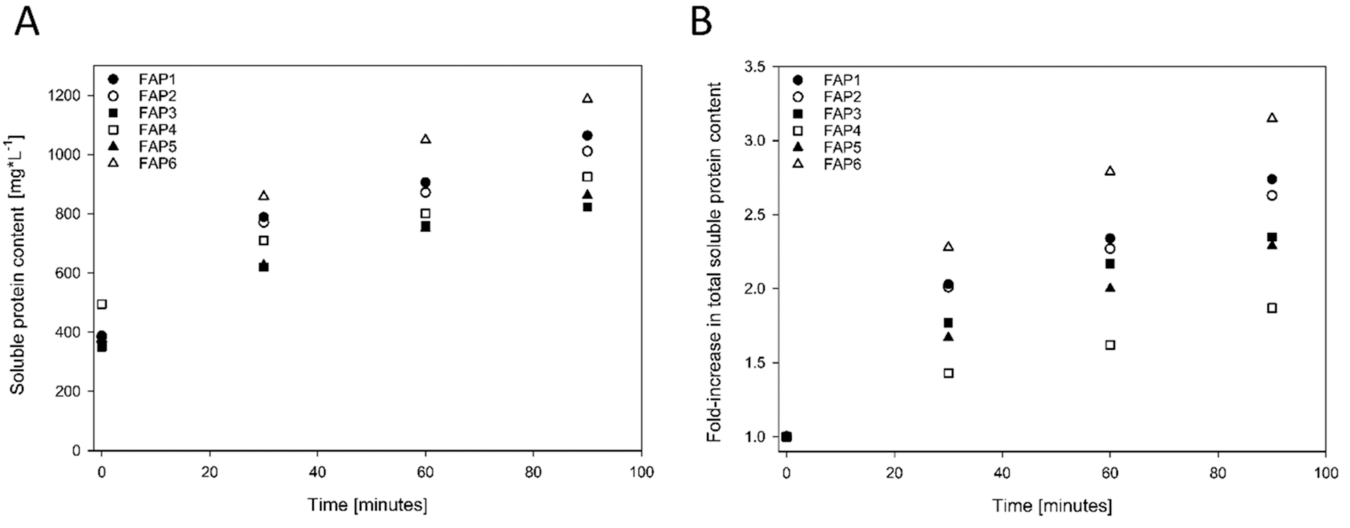

Figure 5. Hydrolysis of the animal feed matrix by each FAP at 41 °C and pH 3.6. Soluble protein content (A) in the animal feed suspension during enzymatic hydrolysis. Fold-increase (B) in the soluble protein content of the hydrolyzed animal feed suspension by each FAP. The values reported are the averages of triplicate measurements; the standard deviation was ≤5%.

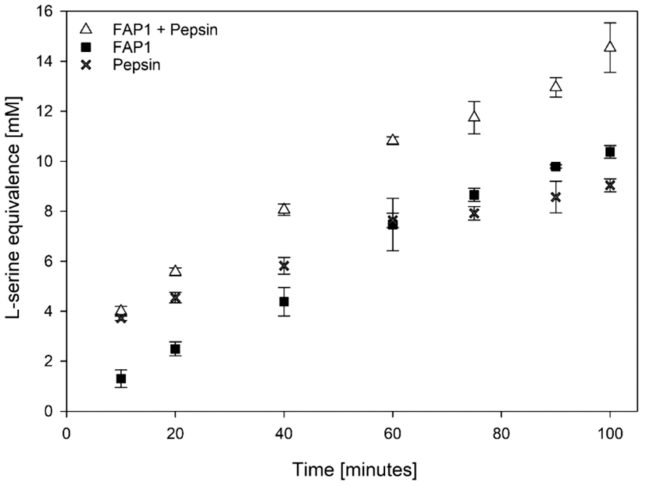

Figure 6. Liberation of L-serine equivalents during the hydrolysis of SPI (3.8% w/v) by FAP1 and pepsin at an E/S of 1:33. The reported values are the averages of triplicate measurements.

Aspergillus are the most commonly used. *Aspergillus* species are only one group among a large number of eukaryotes now cataloged in databanks as producers of proteolytic enzymes. The genus *Aspergillus* includes over 200 species.[31] Around 20 species have been reported as causative agents of opportunistic infections in humans. Among these, *A. fumigatus* is the most commonly isolated species, followed by *Aspergillus flavus* and *A. niger*. In this study, six putative aspartic endopeptidases were selected based on sequence homology from a variety of thermophilic/thermotolerant and mesophilic species of *Aspergillus, Talaromyces, and Thielavia* (Table 1).

Production of Recombinant FAPs in *K. phaffi* and Their Subsequent Biochemical Characterization. Since enzymes expressed in *K. phaffii* frequently exhibit N-linked glycosylation,[32] the FAPs were deglycosylated. Deglycosylated FAP1 and FAP4 resolved into sharp homogeneous bands while FAP6 appeared still slightly glycosylated (Figure 1). This could be due to the occurrence of a different form of glycosylation within FAP6, such as O-linked glycosylation. All of the FAPs had molecular weights within the range of 30−45 kDa which is consistent with the usually reported molecular weights of aspartic endopeptidases.[9,33,34] A holistic comparison between different production strategies could not be made since self-reported titers of heterologous aspartic endopeptidases expressed recombinantly in *K. phaffii* could not be found in most recent literature.

Enzymes that can withstand temperatures between 45 and 120 °C are typically known as thermostable enzymes.[35] FAP4 and FAP6 were most active at 40 °C. While FAP4 lost almost 50% activity at 50 °C, FAP6 managed to retain >90% of its activity at the same temperature. Surprisingly, both FAP4 and FAP6 were able to tolerate continued exposure to temperatures between 40 and 60 °C and were able to exhibit 60 and 100% activity when returned to 40 °C. FAP4 had a pH optimum of 3.0, while FAP6 had a pH optimum of 4.0. While FAP4 lost ∼90% of its activity at pH 2.5, FAP6 retained ∼60% of its activity at the same pH. Both FAPs demonstrated 20−100% proteolytic activity within the pH range of 3.0−6.0. A 47.5 kDa FAP with a pH optimum of 2.5 was purified from *A. niger*. The FAP from *A. niger*, however, was reported to possess a very strict operational pH range; it lost 50% of its activity at pH 3.0 and >80% activity below pH 3.0. It had a reported temperature maximum of 50 °C and it lost ∼50% of its activity after a 30 min exposure to 50 °C.[36] FAP1 and FAP5 demonstrated the highest proteolytic activity at 50 °C and were able to withstand extended exposure to that temperature without losing more than 10% of their activity. While FAP1 and FAP5 demonstrated 40−65% activity at 60 °C during brief exposure time, both FAPs lost between 80−90% of their activity after 20 min of exposure to 60 °C. This result was expected for FAP5 since it came from the mesophilic fungi,[37] *A. niger*. FAP1, however, was identified in *T. terrestris*—one of a limited number of eukaryotic species that are classified as thermophiles. This made FAP1's low tolerance to a moderately high temperature of 60 °C unexpected. A 40 kDa FAP (named TlAPA1), isolated from the thermophilic fungus *Talaromyces leycettanus*, however, was recently reported to possess a similar thermostability. TlAPA1 lost >50% activity after only a 10 min exposure to 60 °C.[38] FAP1 had a pH optimum of 3.5, while FAP5 had a pH optimum of 3.0. Both FAPs retained 40−100% activity within the pH range of 3.0−5.5. A 47 kDa FAP with similar pH optimum, temperature maximum, and thermostability was isolated from *Aspergillus oryzae*.[39] FAP2 and FAP3 demonstrated highest proteolytic activity at 60 °C; however, both the FAPs lost 30−40% of their activity during sustained exposure. Both FAP2 and FAP3 were able to tolerate continued exposure to 50 °C and demonstrated >75%, <80% and >80%, <90% activity at 50 °C, respectively. Both FAP2 and FAP3 had a pH optimum of 4.0, while both maintained 20−100% activity within the pH range of 2.5−6.5. A 45.8 kDa FAP was recently isolated from *T. reesei* with a pH optimum of 4.0 and a temperature maximum of 50 °C. The FAP retained ∼80% of its activity at 60 °C.[21] A 50 kDa FAP was isolated from *A. niger* with an optimum pH of 3.5 and a temperature maximum of 60 °C.[40] A 41 kDa FAP with a similar pH optimum and temperature maximum was isolated from *A. oryzae*; however, it was only stable at temperatures up to 35 °C.[41]

The variability of biochemical characteristics among FAPs from different fungal sources indicates that the choice of an endopeptidase to be employed under acidic conditions needs to be carefully considered to ascertain the optimal combination of pH, temperature, and stability before employing it for an industrial process. All of the FAPs demonstrated pH stability, which makes them suitable candidates for application in the animal feed industry since feed undergoes various pH fluctuations during its passage through the gastrointestinal tract.

An industrially relevant enzyme needs to possess robust tolerances toward chemical species that might be encountered in complex environments, such as food and feed. A holistic comparison of the six recombinant FAPs with the behavior of most of the above-mentioned FAPs under the influence of metal ions, organic solvents, chaotropes, and reducing agents could not be made since relevant comparable data was not found in most recent literature. The six FAPs reported in this study experienced ≤5% reduction in their proteolytic activities in the presence of EDTA, urea, DTT, and β-mercaptopethanol at concentrations of up to 10 mM. The FAP isolated from *T. reesei* behaved similarly except that its activity declined ∼30% in the presence of 1 mM DTT[21] whereas the activity of the FAP isolated from *Rhizomucor miehei* was reduced to <50% in the presence of 1 mM β-mercaptoethanol.[42] The FAP from *T. leycettanus* lost ∼90% of its activity in the presence of 5 mM β-mercaptoethanol.[17] The six FAPs reported in this study

experienced a reduction in their activities in the presence of organic solvents at a concentration of 10% (v/v). The only exception to this observation was an apparent increase in the activity of FAP3 and FAP6 in the presence of DMF and DMSO. All of the tested metals ions at 1 mM concentration had no significant impact on the activities of the FAPs; however, only FAP3 lost ∼30% of its activity in the presence of 1 mM zinc ions. Even Cu^{2+} was unable to completely inhibit the FAPs at 10 mM (∼20% proteolytic activity retention). Most notably, none of the tested metal ions enhanced the proteolytic activities of any of the FAPs reported in this study. Zn^{2+} and K^{+} at 1 mM concentration reduced the activity of the FAP from *R. miehei* by ∼30%.[42] As noted in a previous study by our working group,[21] proteolytic activities of some FAPs are reportedly decreased in the presence of metal ions while other FAPs experience the opposite effect. Different conformational changes that arise when these metal ions bind with some amino acids in these FAPs could be the reason behind the observed enhancement of the proteolytic activity of some FAPs and the inhibition of others.[43]

The tolerance of the six FAPs reported in this study toward monovalent and divalent metal ions, organic solvents, and reducing agents combined with their working pH and temperature ranges would make them viable candidates for exogenous and endogenous applications in complex environments such as the hydrolysis of proteins in digestive tracts of animals, production of bioactive peptides from food proteins, and perhaps even acidic whey protein hydrolysis, where acidic pH values are predominant.

Proteolytic Efficiency of the FAPs, Effects of Potential Product Inhibition, and Evaluation of the Cleavage Preferences of FAP1 and FAP4. To achieve an industrially competitive hydrolysis, one important consideration is the enzyme's activity-to-cost ratio. This is determined not only by the enzyme's specificity toward the chosen substrate but also by the enzyme-to-substrate ratio (E/S) utilized for hydrolysis. In this study, SPI was hydrolyzed by the FAPs at an E/S of 1:700 which is lower than the E/S ratios reported in the majority of other studies cited in this work. Recently, SPI was hydrolyzed by a FAP purified from *A. niger* at an E/S of 3:5.[44] The hydrolytic potential of a FAP purified from *T. reesei* was investigated for food and feed proteins at an E/S ratio of 1:700.[21] Our rationale behind using an E/S ratio on the lower end of the spectrum was to simply demonstrate the applicability of the FAPs even at relatively small dosages.

Inhibition of peptidases by their hydrolysis products is not an uncommon phenomenon. Product inhibition of an enzyme is the consequence of the formation of a nonreactive, stable enzyme−product complex due to the accumulation of peptides as they're liberated during protein hydrolysis.[45] An industrially relevant downside of product inhibition is decreased productivity during batch hydrolysis.[46] Product inhibition of an alkaline peptidase from *Bacillus lentus*, various peptidases present in Flavourzyme, and a FAP from *T. reesei* have been reported.[21,46−48] Most native and recombinant FAPs reported in the literature are not evaluated for the phenomenon of product inhibition. To make a well-informed assessment of the feasibility of upscaling an enzyme's application, it is important to determine whether or not the enzyme is product-inhibited. Soluble peptides (SPH) prepared by Alcalase-mediated hydrolysis of the soy protein isolate were used as potential inhibitors for the FAPs reported in this study. Product inhibition was evaluated using the synthetic substrate "azocasein." This assay was employed because it is based on the release of azo-derivatized casein peptides in the supernatant. Thus, nonderivatized SPH peptides (serving as inhibitors) do not interfere with the absorbance measurement of the assay at 450 nm. Since enzymes' affinities toward synthetic substrates often differ from their affinities toward native substrates, product inhibition data obtained from the hydrolysis of a synthetic substrate cannot be extrapolated to application-based evaluation of native substrates. However, K_m and K_i values obtained from such investigations can still provide insights into a peptidase's proteolytic activity. In order to investigate the potential cause(s) behind the difference between the degree of hydrolysis (DH) yielded by FAP1 and FAP4, the resulting SPI peptides were analyzed via mass spectrometry coupled to liquid chromatography. To determine whether or not the observed difference in the DH was a simple consequence of divergent cleavage specificities of the two peptidases, the N and C terminal amino acids were analyzed to identify P1 and P1' residues and their frequency of occurrence was quantified. The cleavage preference of both FAP1 and FAP4 appeared to be nonspecific and similar to each other. Neither of the FAPs showed any preferential bias toward polar (charged or uncharged) or nonpolar amino acids. Since the DH increased after the sequential addition of FAP1, but did not increase after the sequential addition of FAP4, it would be reasonable to hypothesize that FAP4 was product-inhibited more than FAP1. It would appear that simplistic application of steady-state kinetic parameters, such as K_m or K_i, does not permit for conclusions to be drawn about application-specific data, such as the degree of hydrolysis by the FAPs. The DH achieved by the FAPs reported in the study was comparable to the DH achieved by the FAP isolated from *T. reesei*.[21] Even though there are very few commercial fungal aspartic peptidases available, application of FAPs has recently gained more interest in the food industry.[17,42,44,49] A novel aspartic peptidase from *R. miehei* CAU 432 was expressed in *K. phaffii* and applied for meat tenderization. This FAP was effective in the tenderization of pork as well as the preparation of turtle peptides.[42] In a previous study published by our group, the application of a purified FAP from *T. reesei* showed promising results in hydrolyzing SPI and increasing the soluble protein content of animal feed.[21]

Since peptidases used in animal feed are applied to substrates with compositions that are often not fully known or difficult to control, data from the hydrolysis of relatively pure protein substrates cannot be reliably extrapolated to the hydrolysis of animal feed. Starch and fat present in animal feed can interfere with peptidase activity by reducing protein accessibility. Starch may form complexes with proteins and fat may create hydrophobic barriers, both of which could hinder effective hydrolysis. In the current study, the application of six recombinant FAPs was tested in an *in vitro* assay to evaluate their hydrolytic potential in animal feed hydrolysis and to compare it to their hydrolytic performance against relatively pure SPI. The hydrolyses were carried out at 41 °C and pH 3.6 to mimic the digestive tract of chickens.[29] It is worth noting that only FAP4 and FAP6 had temperature-maximums of 40 °C whereas the remaining FAPs demonstrated ≤84% ≥ 50% proteolytic activity. All of the FAPs except FAP4 had more than doubled the content of soluble protein inside the feed suspension after 60 min of hydrolysis, while FAP6 had even tripled the soluble protein content by the end of 90 min. Interestingly, the hydrolytic performance of the FAPs deviated

slightly from their observed efficiency during the hydrolysis of SPI. FAP5 and FAP4, however, still performed similarly poor compared to others. The observed efficiency of animal feed hydrolysis by the FAPs was as follows: FAP6 > FAP1 > FAP2 > FAP3 > FAP5 > FAP4, whereas the observed efficiency of SPI hydrolysis by the FAPs was as follows: FAP1 > FAP3 > FAP2 > FAP6 > FAP5 > FAP4. This indicates that the peptidases are susceptible to influences by nonproteinaceous components present inside animal feed.

Animal feed constitutes 60−70% of the overall expenses in livestock and poultry production systems.[8] To optimize the utilization of animal feed, it is crucial to minimize losses resulting from inefficient protein digestion by the animal. Yu et al.[11] conducted a study to investigate the impact of incorporating peptidases into the diets of broiler chickens, encompassing both high and low protein corn-soy feeds. The study revealed that the inclusion of a single peptidase not only enhanced feed conversion rates in broiler chickens but also promoted weight gain. An endopeptidase must demonstrate an improved degree of hydrolysis (DH) of feed proteins over hydrolysis by gastric pepsin alone to be viable in the animal feed industry. The DH experienced an ∼45% increase when SPI was hydrolyzed by pepsin and FAP1 simultaneously. Studies have indicated that incomplete development of broiler chicken's gastrointestinal tract (GIT) during the initial days after hatching, combined with suboptimal endogenous enzyme secretion and accelerated passage time of feed through the GIT, results in decreased nutrient uptake.[50] Exogenous endopeptidases could complement the animal's endogenous enzyme secretions and supplement any insufficiency.

These findings alongside others highlighted in this study underscore the potential of aspartic endopeptidases as a valuable yet often overlooked tool for hydrolyzing proteins, specifically animal feed proteins, particularly in acidic environments. FAP1 and FAP6, owing to their robust biochemical characteristics, appear to be likely candidates for further research, which is warranted to validate the broad applicability of FAPs in the food and animal feed industries. While preliminary *in vitro* results indicate promising applications, particularly in the hydrolysis of industrial proteins under acidic conditions, large scale *in vivo* animal trials need to be conducted to evaluate the efficacy of aspartic endopeptidases when supplemented to animal feed either by themselves and/or in combination with other enzymes such as phytases and carbohydrases.

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Author Contributions

T.E. and K.O. supervised the research. T.S. and D.H. designed the synthetic genes. F.K. and H.K. carried out the MS analysis and wrote the relevant methodology section. All other experiments were performed by U.A. U.A. wrote and finalized the manuscript. All authors provided critical feedback to the manuscript. K.B. provided critical feedback during the review process. All authors read and approved the manuscript.

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

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