JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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PsoP1, a Milk-Clotting Aspartic Peptidase from the Basidiomycete Fungus *Piptoporus soloniensis*

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ABSTRACT: The first enzyme of the basidiomycete *Piptoporus soloniensis*, a peptidase (PsoP1), was characterized after isolation from submerged cultures, purification by fractional precipitation, and preparative native-polyarylamide gel electrophoresis (PAGE). The native molecular mass of PsoP1 was 38 kDa with an isoelectric point of 3.9. Similar to chymosin from milk calves, PsoP1 showed a maximum milk-clotting activity (MCA) at 35−40 °C and was most stable at pH 6 and below 40 °C. The complete inhibition by pepstatin A identified this enzyme as an aspartic peptidase. Electrospray ionization−tandem MS showed an amino acid partial sequence that was more homologous to mammalian milk clotting peptidases than to the chymosin substitute from a fungal species, such as the Zygomycete *Mucor miehei*. According to sodium dodecyl sulfate−PAGE patterns, the peptidase cleaved κ-casein in a way similar to chymosin and hydrolyzed β-casein slowly, as it would be expected from an efficient chymosin substitute.

KEYWORDS: *Piptoporus soloniensis*, milk clotting, β- and κ-casein, aspartic peptidase, preparative PAGE

INTRODUCTION

Among the vast number of peptidases used in the food industry, aspartic peptidases, such as chymosin (EC 3.4.23.4) used in cheese-making, are particularly well investigated.[1] Milk clotting by chymosin occurs essentially by cleaving the Phe_{105}−Met_{106} bond of κ-casein, resulting in a release of short hydrophilic glycopeptides (106−169 residues), which dissolve in the whey. *para*-κ-Casein becomes positively charged in neutral milieu. As a result, the repulsive forces between casein micelles decrease, thereby causing aggregation of caseins.[2] The scarcity of traditional rennet, the increase in world cheese production, ethical considerations associated with the slaughtering of milk calves, the rejection of consumers of genetically modified enzymes, and the increase of vegetarian life-styles have promoted the research for alternative coagulants. Plant sources, with few exceptions, such as *Cynara* flower extracts, showed a broader peptidolytic activity, causing extensive digestion of curd, off-flavor, impaired taste, and a pasty texture.[3] Using microbial enzymes, an extensive cleavage of casein, because of their lower specificity and high thermal stability, often resulted in a loss of protein and off-flavor and bitter peptide formation.[2]

Different trials were made to isolate milk-clotting peptidases from alternative sources, such as basidiomycetes.[4−7] Milk-clotting enzymes (MCEs) from *Irpex lacteus* and *Laetiporus sulphureus* were suggested as calf rennet substitutes.[5,8] In a previous paper,[9] the peptidolytic activities of 28 basidiomycetes were screened using different nutrient media. Among the tested basidiomycetes, more than half (16 species) showed milk-clotting activity (MCA). Among the more potent candidates, *Piptoporus soloniensis* (Pso, *Sshirokaimen take*), a close relative of *Piptoporus betulinus* (edible birch polypore), attracted particular attention. This brown-rot fungus combined acceptable growth in submerged culture with high specific clotting and low peptidolytic activities. The focus of the present study was on the purification and physicochemical characterization of its milk-clotting peptidase, PsoP1.

MATERIALS AND METHODS

Chemicals. Ammonium persulphate, ammonium sulfate, bromophenol blue, chymosin, 1,4-dithiothreitol (DTT), ethylenediamine tetraacetic (EDTA), glycerol, glycine, MES, pepstatin A, Rotiphorese 40 (37, 5:1), Rotiphorese 40 (29:1), skim milk powder (∼36% protein and ∼50% lactose), sodium dodecyl sulfate ultra pure (SDS), tetramethylethylenediamine (TEMED), Tris, and Tris-HCl were purchased from Karl Roth (Karlsruhe, Germany). Antipain, azo-casein, bovine serum albumin (BSA), β- and κ-caseins from bovine milk, L-histidine, phenylmethanesulfonyl fluoride (PMSF), rennet from *Mucor miehei* type II, and ultra low range molecular mass marker M 3546 (MW 1060−26600 Da) were from Sigma (Steinheim, Germany). D-(+)-Glucose monohydrate, L-asparagine-monohydrate, potassium dihydrogen phosphate, yeast extract, magnesium sulfate, and Coomassie Brilliant Blue R-250 were purchased from Merck chemicals (Darmstadt, Germany). Calcium chloride-2-hydrate was purchased from Riedel-de Haën (Seelze, Germany). All blue prestained standard was obtained from Bio-Rad (Hercules, United States). Servalyt 40% was from Serva (Heidelberg, Germany)

Handling of Cultures. Pso (CBS, Nr. 279.55) was purchased from the Centraalbureau voor Schimmelcultures (CBS), Netherlands. Cultivation conditions of Pso were described previously by Abd El-Baky et al.[9] Pso was maintained on agar plates (30.0 g/L glucose-monohydrate; 4.5 g/L asparagine-monohydrate; 1.5 g/L KH_2PO_4; 0.5 g/L $MgSO_4$; 3.0 g/L

Received: June 3, 2011
Accepted: August 22, 2011
Revised: August 15, 2011
Published: August 22, 2011

dx.doi.org/10.1021/jf2021495 | J. Agric. Food Chem. 2011, 59, 10311–10316

yeast extract; 15.0 g/L agar−agar; and 1.0 mL/L trace metal solution containing 0.005 g/L $CuSO_4 \cdot 5H_2O$, 0.08 g/L $FeCl_3 \cdot 6H_2O$, 0.09 g/L $ZnSO_4 \cdot 7H_2O$, 0.03 g/L $MnSO_4 \cdot H_2O$, and 0.4 g/L EDTA). The pH of the medium was adjusted to pH 6 with 1 M NaOH prior to sterilization. To prepare precultures, 1 cm × 1 cm agar plugs from the mycelium edge were inoculated into a 300 mL Erlenmeyer flask containing 100 mL of standard nutrient liquid (SNL) and homogenized using an Ultra Turrax (Miccra Art, Germany). Cultures were kept for 7 days at 24 °C on a rotary shaker (Infors, Switzerland) at 150 rpm, and then, 20 mL of the precultures was transferred into a 500 mL Erlenmeyer flask containing 250 mL of fresh SNL. The main cultures were cultivated for 18 days on 24 °C at 150 rpm and harvested with 0.56 U/mL MCA.

Assay for Determination of MCA. MCA was measured according to Arima et al.,[10] based on the visual evaluation of the appearance of the first clotting aggregates. A 10% skim milk solution containing 10 mM $CaCl_2$ was prepared, and the pH was adjusted to pH 6. The enzyme solution as well as the skim milk were preincubated at 35 °C for 10 min. Afterward, 0.2 mL of enzyme solution was mixed with 2 mL of the substrate in a test tube. The coagulation point was determined periodically at 35 °C by manual rotation of the test tube. A control was assessed by thermal activation of enzyme solution at 95 °C for 10 min before mixing with the skim milk. One milk-clotting unit was defined as the amount of enzyme that clotted 1 mL skim milk in 1 min.

Assay of Peptidolytic Activity (PA) and Protein Content. The PA was determined according to Iversen and Jorgensen[11] using azocasein as a substrate. The reaction mixture was made up using 0.2 mL of 2.5% azocasein (w/v) in 0.1 M potassium phosphate buffer, pH 6, and 0.275 mL of 0.1 M potassium phosphate buffer, pH 6, to which 0.025 mL of the enzyme solution was added. The reaction was carried out at 35 °C for 20 min and stopped with 1 mL of 10% trichloroacetic acid (TCA). The tubes were kept on ice for 10 min and centrifuged at 12000*g* for 10 min. The absorbance of the supernatant was read at 366 nm. A blank was prepared by pipetting the enzyme after the addition of the TCA. One PA unit was defined as the amount of enzyme that increased the absorbance of the sample at 366 by one in 1 min. The protein content of the fractions obtained after purification steps was estimated by measuring the absorbance at 750 nm using Lowry method.[12]

Purification of the Enzyme. The culture broth of Pso was centrifuged at 5000*g* for 40 min and 4 °C to pellet the mycelium. The culture supernatant (160 mL) was first gradually saturated with ammonium sulfate. The precipitate was dissolved in histidine-MES 30 mM buffer, pH 6.1, washed three times, and concentrated using Vivaspin-15 concentrator MWCO 3 kDa (Sartorius, Göttingen, Germany). The second purification step was performed using preparative native-polyacrylamide gel electrophoresis (PAGE), model 491 Prep Cell Biorad (Hercules, United States). One milliliter of the concentrated sample was mixed with 1 mL of sample buffer containing 40% glycerol in 3 mM histidine-MES buffer, pH 6.1, and separated on a 14% gel (8 cm gel length, 8.2 cm^2 gel surface area), at 5 W constant power and 4 °C with a flow rate of 0.75 mL/min of 50 mM potassium phosphate, pH 6, as the elution buffer. Fractions (2.5 mL) were collected and checked for MCA.

Characterization of PsoP1. *Temperature Optimum and Thermostability.* To investigate the optimal temperature for PsoP1, MCA was assayed between 30 and 70 °C. The temperature, at which maximum MCA was observed, was set to 100%. The thermal stability of PsoP1 was determined by incubating the enzyme at 30−75 °C for 30 min. After incubation, residual MCA was measured. The nonincubated PsoP1 activity was set to 100%.

Effect of pH on the Activity and Stability. The effect of pH on MCA was tested by assaying MCA in the range from pH 5.5 to 7.5. The pH at which the maximum clotting activity was observed was set to 100%. For pH stability, the enzyme was diluted 1:1 in the following buffer solutions (0.1 M), glycine-HCl (pH 3), sodium acetate (pH 4−5), sodium phosphate (pH 6−8), and glycine-NaOH (pH 9−10), respectively, and incubated at 25 °C. After 22 h, residual MCA was determined. The nonincubated enzyme activity was set as 100%.

SDS-PAGE and Preparative Isoelectric Focusing (P-IEF). Active fractions eluting from the preparative native-PAGE were analyzed by SDS-PAGE, 12% gels, and stained with silver. To determine the isoelectric point of the enzyme, P-IEF was performed using Rotofor cell system, Bio-Rad (Hercules, United States). The conditions were as follows: 0.5% Servalyt 40% (pH 2−4) and 12 W constant at 4 °C for 4 h. The pH of the fractions was measured using a Qph 70 pH-meter (Merck, Germany).

Molecular Mass. The native molecular mass of the enzyme was estimated on a Superdex 75 column, 10 mm × 30 mm from GE Healthcare (Buckinghamshire, United Kingdom), equilibrated with 50 mM sodium phosphate buffer, pH 6.2, containing 150 mM NaCl, with a flow rate of 0.5 mL/min. Eluted fractions were checked for MCA. Estimation of molecular mass was done by means of a calibration with standard proteins.

Inhibition Study. To classify PsoP1, the enzyme was incubated for 15 min at 30 °C with different inhibitors: 0.01 mM antipain (serine and cysteine peptidases inhibitor), 1 mM EDTA (metallopeptidase inhibitor), 0.02 mM pepstatin A (aspartic peptidase inhibitor), and 0.1 mM PMSF (serine peptidase inhibitor). Residual MCA was determined, and MCA of the enzyme without incubation with inhibitors was set to 100%.

Effect of $CaCl_2$ on MCA. The substrate, 10% skim milk, was equilibrated at different concentrations of $CaCl_2$ (0−0.5 M) for 5 min at room temperature; then, the enzyme was added, and MCA was determined. Zero $CaCl_2$ concentration was set to 100% of enzyme activity.

Hydrolysis of κ and β-Caseins by PsoP1. Hydrolysis of κ-casein was examined by dissolving bovine κ-casein in 100 mM sodium phosphate buffer, pH 6.5, to a final concentration of 3 mg/mL. PsoP1 or chymosin (1.7 U/mL) was added to the κ-casein substrates at a ratio of 0.5:10 mL (v/v), and the reaction was allowed to proceed at 35 °C. Aliquots were taken periodically, and the reaction was stopped by heating at 95 °C for 10 min. Samples were applied to 16% SDS-PAGE gels. β-Casein degradation was carried out as described previously by Abd El-Baky et al.[9] A solution of 0.1% (w/v) β-casein in 50 mM potassium phosphate buffer, pH 6, was prepared. Thirty microliters of PsoP1 or chymosin (1.3 U/mL) was mixed with 1.5 mL of β-casein solution and incubated at 35 °C. During the incubation, aliquots (0.2 mL) were taken periodically. The reaction was stopped by heating at 95 °C for 10 min, and samples were applied to 18% SDS-PAGE acrylamide separating gel and 3% acrylamide stacking gel containing 0.1% SDS. After electrophoresis, the gels were stained with Coomassie Brilliant Blue.

Electrospray Ionization (ESI)-Tandem Mass Spectrometer (MS). Partial peptide sequence of PsoP1 was performed as described by Eisele et al.[13] In short, Coomassie-stained protein bands were excised from SDS-PAGE gels, dried, digested with trypsin, extracted, and measured using a Qtof II MS (Micromass, United Kingdom) equipped with a nanospray ion source. The acquired MS-MS spectra were enhanced and used for the ab initio sequencing of tryptic peptides.

RESULTS AND DISCUSSION

Purification of PsoP1. After the fungal pellets were removed by centrifugation, the protein was precipitated from the supernatant using ammonium sulfate. The fractions from 60 to 80% saturation were dissolved in histidine-MES buffer, pH 6.1, and showed 70% MCA with a specific MCA of 2.33 U/mg and a purification factor of 23 (Table 1). Histidine-MES buffer, 30 mM, pH 6.1, was chosen to keep the enzyme active during the run and to impart sufficient charge of the protein to move through the gel. Using the preparative native PAGE, PsoP1 was purified to electrophoretic homogeneity, showing a single band on a silver-stained SDS gel (Figure 1). Active pooled fractions

Table 1. Purification of a Milk-Clotting Peptidase from Pso by Ammonium Sulphate Precipitation and Preparative Native-PAGE

step	total protein (mg)	total activity (units)	specific activity (U/mg)	yield (%)	purification fold
crude extract	888.25[a] ± 12.51	90.25 ± 2.38	0.102 ± 0.001	100	0
ammonium sulfate	27.06 ± 0.90	63.14 ± 1.75	2.33 ± 0.10	69.96 ± 2.74	22.84 ± 1.29
preparative native-PAGE	2.75 ± 0.13	23.37 ± 1.07	8.50 ± 0.37	25.89 ± 1.10	83.33 ± 3.86

[a] Values represent the average ± standard deviation (SD) (n = 3).

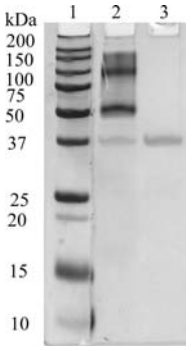

Figure 1. SDS-PAGE of PsoP1 purification steps. Molecular mass marker (1), precipitate after ammonium sulfate treatment (2), and purified PsoP1 after preparative native-PAGE (3).

Table 2. Specific MCA of the Pso Milk-Clotting Peptidase and *Mucor* Rennet[a]

enzyme	MCA (U/mL)	PA (U/mL)	MCA/PA ratio
Pso	2.55[b] ± 0.12	0.05 ± 0.003	51.0 ± 2.21
M. miehei	3.51 ± 0.10	0.045 ± 0.002	78.0 ± 2.71

[a] Both enzyme solutions were adjusted to 0.3 mg/mL protein. [b] Values represent averages ± SDs (n = 3).

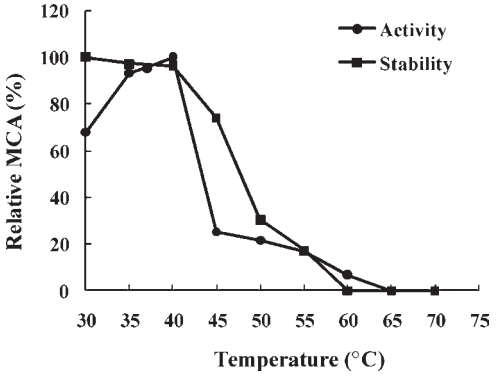

Figure 2. Effect of temperature on MCA (●) and stability (■) of PsoP1 and 100% equals to 2.55 U/mL. SD values were less than 5% (n = 3).

exhibited a specific MCA of 8.50 U/mg with a recovery of 26% and a purification factor of 83 (Table 1).

MCA. Table 2 compiles the clotting and peptidolytic activities of PsoP1 as compared to the microbial reference enzyme from *M. miehei*. The peptidolytic activities were similar for both sources, but PsoP1 showed a somewhat lower MCA/PA ratio, a measure generally regarded as an index of the clotting efficacy. Commercial *Mucor* rennet is supposed to be chemically and genetically altered to obtain a maximum MCA/PA ratio by glycosylation of

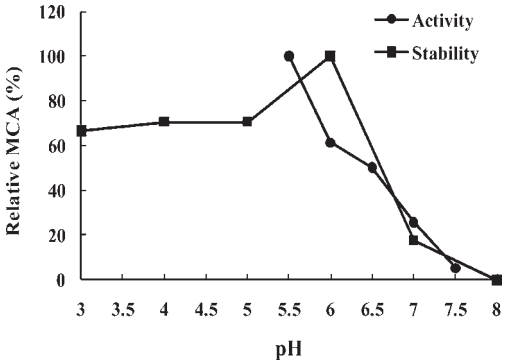

Figure 3. Effect of pH on MCA (●), 100% is equivalent to 2.55 U/mL and stability (■), 100% is equal to 2.15 U/mL, of PsoP1. SD values were less than 5% (n = 3).

Mucor rennet.[14] However, the absolute peptidolytic activities of both enzymes were very low, and thus, small deviations can result in large changes of the MCA/PA ratio. Moreover, data on PA inevitably depend on the substrate used, and different substrates are known to yield different absolute activities. Nonetheless, the results clearly indicated a high clotting activity and low PA of PsoP1.

Temperature Optimum and Thermostability. As shown in Figure 2, PsoP1 exhibited its maximum clotting activity between 35 and 40 °C. This was similar to results of Berridge,[15] who determined the optimum temperature for purified chymosin to be between 30 and 40 °C. On the other hand, MCE from *M. miehei* showed its maximum MCA at 60 °C.[16] The temperature of cheese processing is usually set from 30 to 35 °C. At this temperature, PsoP1 retained its maximum activity. PsoP1 remained stable until 40 °C for 30 min, while it lost 75% of its activity after incubation at 50 °C for 30 min (Figure 2). A complete denaturation was observed at 60 °C. Hence, PsoP1 was also more sensitive to thermal treatment than the commercial clotting enzyme from *Rhizomucor miehei*, which exhibited high stability at 60 °C.[17] As a result, the temperature inactivation characteristics of PsoP1 rather resembled chymosin than most of the microbial sources.

Effect of pH on the Activity and Stability of PsoP1. The highest MCA was observed at pH 5.5 followed by a decrease to pH 7.5 (Figure 3). Other MCEs exhibited the same trend.[18] PsoP1 was stable from pH 3 to 5 and retained about 70% of its activity with a maximum stability at pH 6. At higher pH values, the enzyme lost its activity (Figure 3). Mezina et al.[19] reported that chymosin was likewise more stable at acidic pH values and denatured above pH 6.

Molecular Mass and Isoelectric Point (p*I*) of PsoP1. The native molecular mass of the PsoP1 was estimated by gel filtration (Superdex 75 column) to be 38 ± 1.4 kDa. This result was similar to calf chymosin (36.3 kDa[20]). The p*I* of the PsoP1was 3.9, comparable to that of *L. sulphureus* peptidase (p*I* 3.5[5]). On the other hand, the p*I* of chymosin was 4.5.[20]

Table 3. Effect of Peptidase Inhibitors on MCA of Milk-Clotting Peptidase from Pso

inhibitors	concentration (mM)	relative MCA (%)
control	0	100
antipain	0.01	94.5[a] ± 2.00
EDTA	1	98.5 ± 1.51
pepstatin A	0.02	0
PMSF	0.1	51.1 ± 1.1

[a] Values represent averages ± SDs (n = 3).

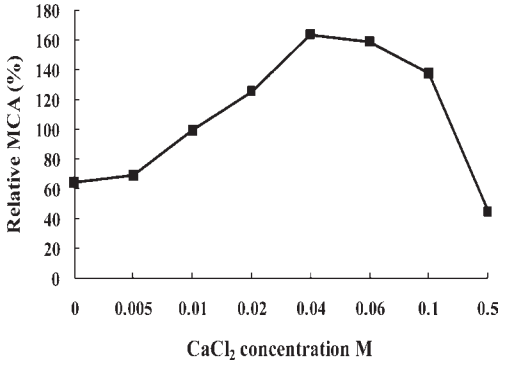

Figure 4. Effect of $CaCl_2$ on MCA of PsoP1 and 100% is the same as 2.55 U/mL. SD values were less than 5% (n = 3).

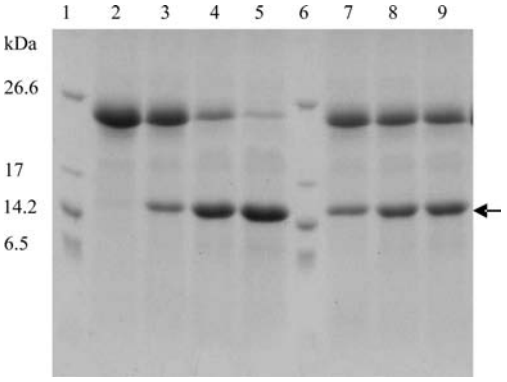

Figure 5. SDS-PAGE pattern of κ-casein hydrolyzed by chymosin and PsoP1. Molecular mass marker (1, 6), nonhydrolyzed κ-casein (2), κ-casein hydrolyzed by chymosin for 5, 15, and 30 min (3−5), and κ-casein hydrolyzed by PsoP1 for 5, 15, and 30 min (7−9); the generated peptide (*para*-κ-casein) is indicated by the arrow.

Inhibition Study. To classify PsoP1, several peptidase inhibitors were employed to identify functional groups at the active site of this enzyme (Table 3). Antipain and EDTA did not alter the activity of the enzyme showing that the enzyme does not belong to metallo- or serine- and cysteine-peptidases. In the presence of PMSF, a serine peptidase inhibitor, PsoP1 lost 51 ± 1.1% of its activity. A complete inhibition was observed in the presence of 20 μM pepstatin A, suggesting the presence of aspartyl residues on the active site. It was reported that aspartic peptidases are inhibited by pepstatin A, a hexapeptide from *Streptomyces* that contains two statin residues, an unusual amino acid.[21]

Effect of $CaCl_2$ on MCA of PsoP1. Calcium is considered as a stimulator for coagulation by creating isoelectric conditions and by acting as an ion bridge between the phosphate moieties of casein micelles.[18] In the case of PsoP1, MCA increased by increasing the concentration of $CaCl_2$. The maximum increase (2.5-fold) was reached at 0.04 M $CaCl_2$ (Figure 4). At concentrations higher than 0.04 M, MCA decreased, probably due to the saturation of negative residues of the micelles in the enzyme substrate mixture.[22] This was in the range of the genuine calcium concentration of cow's milk (around 0.03 M). The distinct response of PsoP1-treated milk to the addition of $CaCl_2$ is in line with the high MCA of this enzyme and is expected to support the gel strength, aggregation rate, and curd firmness in cheese production.

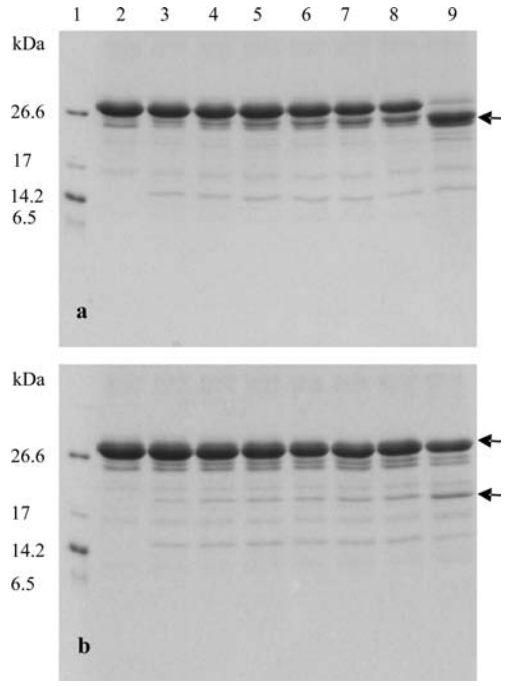

Figure 6. SDS-PAGE pattern of β-casein hydrolyzed by chymosin (a) and PsoP1 (b). Molecular mass marker (1), nonhydrolyzed β-casein (2), and β-casein hydrolyzed for 30, 60, 90, 120, 180, and 240 min and 24 h (3−9); the generated peptides are indicated by arrows.

Hydrolysis of κ-and β-Caseins. Hydrolysis of κ-casein by PsoP1 as well as by chymosin was monitored by SDS-PAGE (Figure 5). After an incubation period of 5 min, both enzymes produced one major band with a molecular mass of about 16 kDa, which is assumed to be *para*-κ-casein. The intensity of the *para*-κ-casein band increased with the incubation time. After 30 min, κ-casein was almost completely degraded by chymosin, while it was more stable toward the action of PsoP1. The slower proteolysis of κ- and *para*-κ-casein is supposed to increase the yield of the curd. In contrast, commercial rennet substitutes of *M. miehei* and *Endothia parasitica* caused an extensive nonspecific hydrolysis of both κ-casein and *para*-κ-casein.[23]

The differences in cheese properties during ripening and storage are mainly caused by the combined effects of the ongoing degradation of α- and β-caseins. Unwanted bitter peptides may arise from their hydrophobic sequences in the course of random hydrolysis.[2] Figure 6 shows the degradation of β-casein by PsoP1 as compared to chymosin. β-Casein was slightly hydrolyzed by both chymosin and PsoP1 after 30 min. The intensity of the generated bands increased during 4 h of incubation. After 24 h, β-casein was completely degraded by the action of chymosin to a

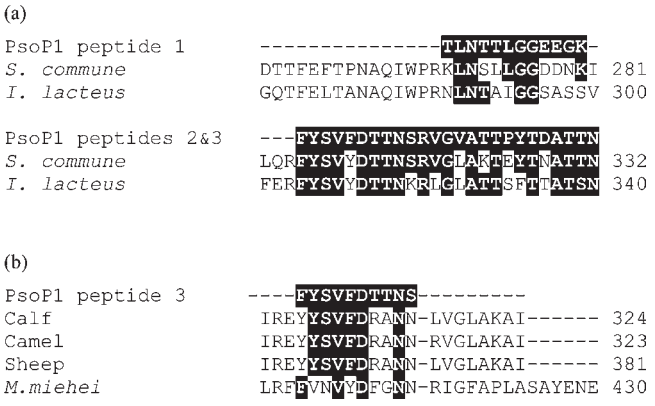

Figure 7. (a) Amino acid sequence alignment of the peptides of the PsoP1 as compared to cDNA-derived amino acid sequence of *Schizophyllum commune*[25] and *I. lacteus*[26] peptidases. Identical amino acids are shaded. (b) Amino acid sequence alignment of single peptide of the PsoP1 as compared to cDNA-derived amino acid sequence of calf prochymosin,[27] camel chymosin,[28] sheep preprochymosin,[29] and *M. miehei*.[30] Identical amino acids are shaded.

major band with a molecular mass of 24 kDa, while it was hardly hydrolyzed by PsoP1. Only one thin band with a molecular mass of 18 kDa was visible even after 24 h.

The desired limited hydrolysis of β-casein by chymosin is well-known.[5] On the other hand, *M. miehei*, *I. lacteus*, and *E. parasitica* milk-clotting peptidases exhibited high PA toward β-casein.[24] The limited PA of PsoP1 is considered as a unique characteristic among the various microbial origins and suggests a particular suitability for the production of cheese.

Partial Peptide Sequences. Three tryptic peptides were identified from the protein band excised from the SDS gel by means of ESI-MS/MS. Homology searches against public databases identified the enzyme as a member of aspartic peptidase A1 family, similar to aspartic peptidases of *Schizophyllum commune*[25] and *I. lacteus*.[26] The N-terminal sequence of the tryptic peptides of PsoP1 showed 50−90% identity to the aspartic peptidase from *S. commune* and 42−82% identity to the one from *I. lacteus* (Figure 7a). *I. lacteus* and Pso are taxonomically related. Both of them belong to the order *Polyporales*, while *Schizophyllum* is a representative of the *Agaricales*. This supported the observation (during the initial screenings) of milk-clotting activities in basidiomycete species from different orders. Alignment of the tryptic peptides of PsoP1 obtained and MCEs of calf,[27] camel,[28] sheep,[29] and *M. miehei*[30] revealed that two of them did not show a good homology with any other source. The third peptide (FYSVFDTTNSR) was surprisingly highly homologous to all mammalian sources but less to *Mucor miehei* (Figure 7b). This agrees with the above findings that some physicochemical features of PsoP1 were closer to calf chymosin than to microbial rennets. Speculations on sequence−property relationships, however, must remain premature, until the full sequence of PsoP1 has become available.

In conclusion, an aspartic peptidase from Pso was purified using a simple two-step purification procedure. The enzyme showed a unique combination of properties: high MCA with a low general PA, moderate temperature stability, maximum activity at a weakly acidic pH, and a very slow peptidolysis of β-casein. As a result, the biotechnology of basidiomycetes represents an inexhaustible source of MCA. The transfer of peptidase genes into heterologous hosts, such as *Escherichia coli*, was successful[16] and could offer an alternative route, if the superiority of genetically engineered food enzymes will find wider appreciation by consumers.

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Funding Sources

Financial support of H.A. by the Egyptian Ministry of Higher Education and the DAAD, Bonn, is gratefully acknowledged.

ABBREVIATIONS USED

EDTA, ethylenediamine tetraacetic; ESI, electrospray ionization; MCA, milk-clotting activity; MCEs, milk-clotting enzymes; MS, mass spectrometer; PA, peptidolytic activity; PAGE, polyacrylamide gel electrophoresis; P-IEF, preparative isoelectric focusing; PMSF, phenylmethanesulfonyl fluoride; Pso, *Piptoporus soloniensis*; PsoP1, *Piptoporus soloniensis* milk-clotting peptidase; SDS, sodium dodecyl sulfate

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