Characterization of Proteases from *Rhizopus* Species after Growth on Soybean Protein

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Z. Naturforsch. **52c,** 595–604 (1997); received May 6/July 3, 1997

Soyprotein, *Rhizopus*, Proteases, Tempe, Inhibitors

Culture filtrates of different fungi of the genus *Rhizopus* forming tempe (i.e. traditional Indonesian food) were grown on a soybean protein-raffinose-phytate medium and investigated for protease activity using soyprotein as substrate. All isolates belonging to the species *R. oryzae*, *R. stolonifer*, *R. oligosporus*, and *R. microsporus* var. *chinensis*, formed the well-known *Rhizopus*-pepsin (aspartic proteinase, 35 kD, isoelectric points: 5.9, 5.0, <4) and an additional protease mainly active under alkaline conditions. The new protease (33 kD, isoelectric points: variable and isolate specific) was purified approximately 300-fold and shown to be a serine protease (inhibitor studies). During fungal culture (12–135 h) the aspartic proteinase is expressed first followed by the serine protease. Both proteases are insensitive to the soybean Kunitz and Bowman-Birk inhibitors. The best rate of soyprotein degradation is achieved by the coordinate action of both proteases at pH 6.5. The examined *Rhizopus* isolates differ in the time course and intensity of the expression of the alkaline protease.

Introduction

Tempe (tempe kedelai) is a traditional Indonesian food produced by fermenting soybeans mainly with fungi of the genus *Rhizopus* (*R. oligosporus*, *R. oryzae*, *R. stolonifer*). Two fermentation steps can be distinguished. During the first phase the metabolic activity of lactic acid bacteria decreases the medium pH and thereby improves the growth conditions for *Rhizopus* species in the second phase. The fermentation leads to a nutritional improvement of soybeans regarding fatty acid composition, removal of undesirable components like the α-galactosides, stachyose and raffinose as well as phytate, and an increase in the amount of several vitamins (Nout and Rombouts, 1990; Bisping *et al.,* 1993; Rehms and Barz, 1995). Tempe is a very good source of vegetable protein and it contains approximately 40% protein on a dry matter basis. During tempe fermentation the content of free amino acids increases (Baumann and Bisping, 1995) indicating pronounced protease activity of the fungi. Protein of soybeans and other legumes is considered to be of poor digestibility (Nielsen, 1991). Fungal fermentation of the soybeans leads to protein degradation and this greatly improves their digestibility by man. Therefore, *Rhizopus* isolates with a high proteolytic activity in the early phase of tempe fermentation are of an advantage.

Rhizopus species produce many hydrolytic enzymes such as glycohydrolases, lipases, phytases, and proteases (Nout and Rombouts, 1990). In order to select *Rhizopus* strains with the ability to express high phytase activities, to metabolize the soybean α-galactosides as well as to grow on and to degrade soyprotein, numerous *Rhizopus* isolates obtained from various stages of tempe production were screened (Rehms, 1993). Six isolates belonging to *R. oryzae*, *R. stolonifer*, *R. oligosporus*, and *R. microsporus* var. *chinensis* were chosen for further studies (Rehms and Barz, 1995). For investigations on proteases they were cultivated in a liquid soyprotein-raffinose-phytate medium, a system comparatively similar to soybean fermentation conditions.

The work reported here was designed to study the protease system of tempe forming *Rhizopus* strains during growth on soyprotein with regard

Abbreviations: ACP, acidic protease; ALP, alkaline protease; BBI, BOWMAN-BIRK inhibitor; KI, KUNITZ inhibitor; IEP, isoelectric point; MSP, malt extract-soypeptone medium; SRP, soyprotein-raffinose-phytate medium; EDTA, ethylendiamine tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride.

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to protease activity, number of enzymes and their multiple forms as well as degree of soyprotein digestion during tempe fermentation. Furthermore, the interactions between *Rhizopus* proteases and the well-known soybean proteinase inhibitors were investigated. These inhibitors are the Kunitz inhibitor (KI, ~20 kD) which inhibits trypsin and the "double-headed" Bowman-Birk inhibitor (BBI, ~8 kD) which can inhibit chymotrypsin and trypsin simultaneously.

Wang and Hesseltine (1970) described an acid protease from *R. oligosporus*. The best-investigated *Rhizopus* species regarding protease is *R. chinensis*, from which an aspartic proteinase, *Rhizopus* pepsin (EC 3.4.23.21) (Rawlings and Barrett, 1993), has been characterized (Fukumoto *et al.*, 1967; Tsuru *et al.*, 1969). Two equal sized forms of this enzyme have been characterized and sequenced (Graham *et al.*, 1973; Takahashi, 1988). *Rhizopus* pepsin has also been a favorite subject for analyses of molecular structures of aspartic proteases (Moa, 1991; Parris *et al.*, 1992).

Soyprotein as a substrate is rarely used for detecting proteolytic activity. However, soyprotein is more suitable than casein for analysis of the degradation process of soybean protein by *Rhizopus* proteases during tempe fermentation. The use of soyprotein as a substrate for *Rhizopus* proteases has not yet been reported and the protease isoform expression of most tempe forming *Rhizopus* isolates is so far unknown. Early data on growth of *Rhizopus* species on soybeans reported no enzyme studies (Wang and Hesseltine, 1965). Expression of proteolytic activity during digestion of soyprotein by different endo- and exopeptidases of *Aspergillus* species regarding shoy koji and miso production has been determined (Sekine *et al.*, 1970; Nakadai *et al.*, 1972a, b; Nakadai and Nasuno, 1977).

Materials and Methodes

Fungal strains

The *Rhizopus* isolates Fi, Mala, EN (*R. oryzae* Went & Prinsen Geerligs), Sur (*R. microsporus* van Tieghem var. *chinensis* (Saito) Schipper & Stalpers), CM (*R. stolonifer*) as well as Sama, MS1, and MS5 (*R. oligosporus* Saito) were obtained from the Institute of Microbiology, Münster. They had been isolated mainly from Indonesian tempe, soaking water samples, and tempe inocula. The *Rhizopus* strain NRRL 2710 (*R. oligosporus*) was obtained from the Centraalbureau voor Schimmelcultures, Baarn, Netherlands.

Media

The fungi were grown on a liquid soyprotein-raffinose-phytate (SRP) medium (100 ml/200 ml-flask) with 1% (w/v) soyprotein (alpha-protein, ICN), 1% (w/v) alpha(α)-D-raffinose, and 0.125% (w/v) sodium phytate (both Sigma). Raffinose and phytate were added by sterile filtration after autoclaving the soyprotein for 30 min. After shaking for 15–20 min the medium pH was found to be 6.5 (± 0.1). Most of the soyprotein (approximately 95%) is insoluble. A second cultivation medium, a malt extract soypeptone medium (MSP) contained 4% (w/v) malt extract and 1% (w/v) soypeptone (100 ml/200 ml-flask).

Buffers

The following solutions were used at the relevant pH intervals: pH 1.5: 0.5 M HCl, pH 2–6.5: 0.5 M citric acid-NaOH, pH 6.5–9.0: 0.5 M tris-(hydroxymethyl)-amino-methane (TRIS)-HCl, pH 9.0–12.5: 0.5 M glycine-NaOH, pH 13.0: 0.5 M NaOH.

Investigations on culture filtrate

Media were inoculated with *Rhizopus* spores (culture conditions: 30 °C, 180 rpm on a NEW BRUNSWICK SCIENTIFIC rotary shaker) and flasks were harvested according to Rehms (1993) after 12, 24, 36, 48, 72, and 135 h. The different media samples were utilized for determination of pH values, the degradation status of soyprotein as well as protease detection.

Protease assay with soyprotein

The assay with casein as substrate was modified for determination of proteolytic activity. Alpha(α)-protein (10 mg/ml) was dissolved at pH 8.0 with 40 µM NaOH for approximately 2 h. The test assays containing 150 µl buffer (different pH values, see above), 90–0 µl aqua bidest, 10–100 µl enzyme solution, and 250 µl of the α-protein solution were incubated for 30 min at 37 °C. The reaction was stopped by addition of 500 µl 10% (w/v)

trichloroacetic acid (TCA). The amount of solubilized protein was estimated photometrically at 280 nm and related to a L-tyrosine standard. All readings were corrected for the blanks which were prepared by first mixing the soyprotein solution with TCA and then adding enzyme solution.

For determing pH optima of the proteases the pH range 1.5–13.0 (steps of 0.5 pH units) was chosen using different buffers. Temperature dependence of proteolytic reaction was determined from 0–70 °C in 10- and 5 °C-steps.

Protease assay with azocasein

Sulfanilamide azocasein substrate (Sigma) was used for rapid detection of protease activity during enzyme purification and inhibition tests. Assays described by Sarath *et al.* (1989) were modified. 75 µl azocasein (20 mg/ml, pH 8.0, dissolved in 40 µM NaOH) were added to 50 µl buffer (pH 5.5 for the acidic protease (ACP); pH 9.0 for the alkaline protease (ALP)), 70–0 µl aqua bidest, and 5–75 µl enzyme sample. After incubation for 30 min at 37 °C the reaction was stopped by the addition of 600 µl 10 % (w/v) TCA. 600 µl of the TCA soluble supernatant were mixed with 700 µl NaOH (1 M) and the absorption of liberated dye was measured at 410 nm. For the calculation of ACP activity at pH 5.5 the contribution of ALP activity, also measured at pH 5.5, had to be substracted from the total protease activity.

Enzyme purification

Protein from the culture filtrate was concentrated by acetone (cooled to -20 °C) precipitation (55 % final concentration). The protein pellet was collected by centrifugation (27500×*g*, -5 °C, 30 min) and resuspended in citrate phosphate buffer (50 mM, pH 6.0) for 3 h. Undissolved material was removed by centrifugation (27500×*g*, -5 °C, 30 min) and discarded. The supernatant was desalted on a Sephadex G 25 (PD-10) column (Pharmacia) in citrate phosphate buffer (20 mM, pH 6.0). All subsequent purification steps were performed on a Fast Protein Liquid Chromatography system (FPLC) (Pharmacia, Freiburg, F. R. G.). The concentrated culture filtrate was first applied to a Phenylsuperose HR 5/5 column (maximal 10 mg protein per run) equilibrated with citrate phosphate buffer (50 mM, pH 6.9, 1.7 M ammonium sulfate) and the proteases were eluted with a linear gradient down to 0 M ammonium sulfate (AS) in the same buffer without AS. Protease active fractions were pooled and transferred to 2-[4-(2-hydroxyethyl-1-piperazinyl)]-propanesulfonic acid (HEPES)-NaOH buffer (50 mM, pH 7.6). The active enzyme solutions of 3–4 Phenylsuperose chromatographic runs were loaded onto a Mono Q HR 5/5 column (45 mg protein per run) and eluted with an increasing NaCl-gradient up to 0.5 M in the same buffer. The void volume of the Mono Q chromatography contained the ALP while the ACP bound to the column. The proteins from the void volume were transferred to sodium acetate buffer (50 mM, pH 5.0) and applied to a Mono S HR 5/5 column. The elution was performed with a linear gradient up to 0.25 M NaCl. Proteolytic active fractions of Mono Q and Mono S chromatography runs were separately pooled and used for determination of protease types, isoelectric points (IEP), and other characteristics.

Gel filtration

Molecular weights of native ACP and ALP were determined by gelfiltration with Superdex 75 (High Load, Prep grade Pharmacia) equilibrated with sodium acetate buffer (0.1 M, pH 5.5, 0.5 M NaCl) for ACP and with HEPES-NaOH (0.1 M, pH 7.6, 0.5 M NaCl) for ALP using hexokinase (100 kD), bovine serum albumin (BSA) (67 kD), beta(β)-lactoglobulin (36.5 kD), cytochrome C (12.5 kD) and vitamin B_{12} (1.36 kD) as standards.

Protein concentration

The protein concentration of appropriate samples was determined by Bradford (1976) or by a modified method as described by Sandermann and Strominger (1972) with precipitation by 24% (w/v) TCA solution in presence of deoxycholate (Bensadoun and Weinstein, 1976). BSA was used as a standard.

SDS-PAGE

SDS-PAGE was performed according to Laemmli (1970) on a slab gel of 12.5% acrylamide with a minigel apparatus (Biometra) or on a 10–20% linear gradient gel (1.5 x 120 x 135 mm). Gels with soyprotein samples were stained with Coo-

massie Brilliant Blue G-250 (Neuhoff *et al.*, 1988). Gels containing protease samples were silver stained according to Merril *et al.* (1981). Stained soyprotein bands were scanned by a laser densitometer (Ultroscan XL Laser Densitometer, Pharmacia).

Determination of isoelectric points

IEPs of purified ACP and ALP were determined by chromatofocusing as well as by isoelectric focusing (IEF). Chromatofocusing in the pH interval 7.0–4.0 for ACP multiple forms, and 7.0–4.0 and 9.0–6.0 for ALP multiple forms was carried out on a Mono P HR 5/5 column according to the instruction guide of Pharmacia (LKB Instruction No. 52–1624–00-AD, 1989) using 7.5% (v/v) Polybuffer 74 and 10% (v/v) Polybuffer 96 (both Pharmacia). In such assays protease activities were considered to represent discrete forms if the activity level in the azocasein assay was more than 0.07 ΔE_{410}/30 min * 20 µl (assay conditions) and furthermore when the activity was obtained in more than 1 ml eluate (fraction size: 0,5 ml), and when these results were reproducible.

IEF was performed at a pH interval of 4.0–7.0 (immobiline gel) and 3.0–10.0 (ampholine gel) using 124 x 258 mm GelBond-PAG film and a cooled Multiphor II-2117-System (Pharmacia). Proteolytic activity in the gels was detected by the azocasein assay after cutting the lanes into 3 mm-segments.

Semidry Western blot

For glycoprotein detection SDS-PAGE separated proteases were blotted onto nitrocellulose by a NOVA-BLOT-System and the Multiphor II-System according to LKB Instruction (No. 2117–005/250) and analyzed with the lectin concanavalin (Con) A and horse-radish peroxidase as a reporter enzyme.

Effects of proteinase inhibitors

Inhibitors were purchased from Sigma, Boehringer Mannheim, and Merck. Effects of proteinase inhibitors were checked following manufacturer's guides (Merck Biochem. Service 3.91; Boehringer Mannheim Protease Inhibitors) and Monod *et al.* (1991). Ethylenediaminetetraacetic acid (EDTA), soybean Kunitz inhibitor and soybean Bowman-Birk inhibitor, were dissolved in water and pepstatin A, phenylmethylsulfonyl fluoride (PMSF), plus *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E-64) were dissolved in ethanol. Chymostatin was dissolved in dimethylsulfoxide (DMSO). Cystatin was obtained ready to use in buffer (Boehringer). Depending on the inhibitor, 5–38 µg/ml (concentration in test) ACP and ALP respectively were preincubated (15 min, 35 °C) with different inhibitors (concentrations according to Table I) at pH 5.5 and/or 7.5 in dependence on the inhibitor and protease stability (total volume 125 µl). Subsequent protease assays were started by addition of azocasein dissolved in sodium acetate buffer (0.5 M, pH 5.5) for ACP, and TRIS-HCl (0.5 M, pH 7.5) and glycine-NaOH buffer (0.5 M, pH 9) for ALP respectively. Appropriate controls without inhibitor and with only the inhibitor solvent were run simultaneously.

In vivo soyprotein digestion

The extent of soyprotein degradation during cultivation of *Rhizopus* species in SRP medium was estimated for the insoluble portion by separating the protein grains from the mycelium and determining their dryweight, and for the soluble part by determining the protein concentration according to the modified Lowry method.

In vitro soyprotein digestion

For determination of soyprotein hydrolysis by purified fungal proteases, commercial α-protein, salt-soluble (0.5 M NaCl) soyprotein isolated from cooked and dehulled soybeans (modified method by Horan, 1974), and soybean proteinase inhibitors, respectively, were used as substrates. Incubations were carried out at pH 5.5 with ACP, pH 9 with ALP, and at pH 6.5 with both proteases. For analyses of α-protein digestion α-protein (10 mg/ml test concentration), ACP and/or ALP (each 2.5 µg/ml) were incubated in a volume of 1 ml under sterile conditions at 37 °C, and 50 µl aliquots of the incubation mixture were taken at 0, 12, and 48 h for SDS-PAGE. Appropriate protein and enzyme controls were run simultaneously. Salt-soluble soyprotein was digested for 0, 0.25, 12, and 48 h under the same conditions with 0.93 mg/ml soyprotein, 0.5 µg/ml ACP, and/or 0.5 µg/ml ALP

in the presence of 0.5 M NaCl. For SDS-PAGE 15 µl aliquots were used. For degradation studies Kunitz inhibitor (KI) (0.113 mg KI/ml, Merck) and Bowman-Birk inhibitor (BBI) (0.141 mg BBI/ml, Sigma) were incubated at pH 6.5 (37 °C) with 5.75 µg/ml ACP and 5.1 µg/ml ALP together, and the hydrolysis products were separated on SDS-PAGE after 0, 24, and 48 h incubation.

Results and Discussion

Growth in soyprotein-raffinose-phytate medium and pH value

The studies on soyprotein digestion and protease characterization were performed with 9 selected *Rhizopus* isolates belonging to the species *R. oryzae*, *R. stolonifer*, *R. oligosporus*, and *R. microsporus* var. *chinensis*. The well investigated tempe-producing strain NRRL 2710 (Hesseltine, 1989) was included for comparison. These isolates when cultured on a medium containing soyprotein grew well (200–400 mg dry weight/100 ml after 48 h). But during growth on the malt extract soypeptone medium the *Rhizopus* isolates formed twice to three times as much mycelial mass. During growth on soyprotein the *Rhizopus* isolates caused an increase of pH value from 6.5 to 7.2–7.4, except isolate Sur which lowered the pH in the medium to 5.

In vivo degradation of soyprotein

The pH value of the medium appears to be correlated with the degree of soyprotein degradation. Isolate Fi, which increased the pH value quickly, had no residual insoluble protein after 24 h and only 19 % of the starting protein (when ignoring the amount of protein released by the fungus) were still detectable in contrast to isolate Sur, which needed 135 h for total digestion. Increasing the pH to 7.3 (± 0.1) favors protein solubility (Waggle and Kolar, 1979) and thereby probably the rate of soyprotein degradation by proteases.

Differences in the rate of protein degradation were also correlated with the time course of protease expression by the fungi. After certain time intervals of growth the proteolytic activity in the medium was assayed over the pH range of 1–13. Pronounced differences between the isolates were found. As an example, Fig. 1 compares the proteolytic activity expressed by the isolates Fi and Sur, at one time point (48 h) using the soyprotein assay. Sur exhibited very low protease activity around pH 3 12 h after inoculation (data not shown). After 24 h enzyme activity had increased up to tenfold at pH 3 (3.95 µmol tyrosine/min * flask), and another smaller maximum appeared around pH 5.5 (1.3 µmol tyrosine/min * flask). Wang and Hesseltine (1970) also described protease activity maxima at pH 3 and 5.5 for *R. oligosporus* when grown on wheat flour. Protease activity above pH 7 could not be detected and up to 72 h protease activity in the medium remained constant. However, 135 h after inoculation enzyme activity in the alkaline range with a maximum at pH 10 was found. In contrast, the culture filtrate of iso-

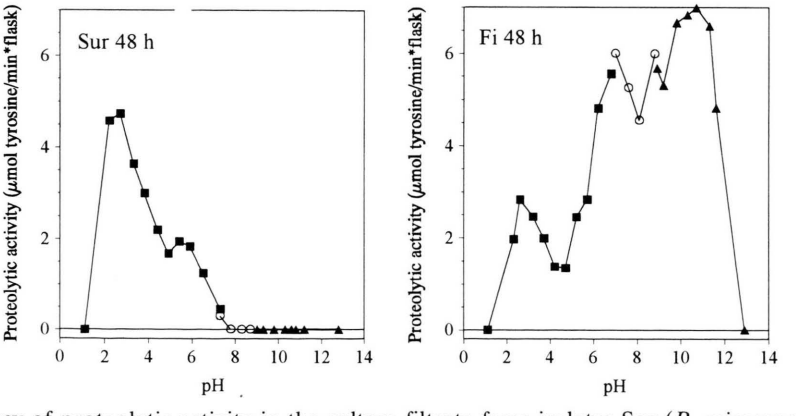

Fig. 1. pH dependency of proteolytic activity in the culture filtrate from isolates Sur (*R. microsporus* var. *chinensis*) and Fi (*R. oryzae*) after growing 48 h on soyprotein-raffinose-phytate medium (SRP). (■) citric acid-NaOH, (○) TRIS-HCl, (△) glycine-NaOH, pH 1,4: 0.5 M HCl, pH 13: 0.5 M NaOH.

late Fi, 12 h after inoculation, showed distinct protease activity at pH 3 (1.7 μmol tyrosine/min * flask) and at pH 5.5 (1 μmol tyrosine/min * flask), and low enzyme activity at alkaline pH (data not shown). After 24 h of growth activity in the alkaline range had increased 13-fold with a broad maximum around pH 9. Enzyme activity at pH 7–11 did not significantly change within the next 2 days. Quite remarkably, after 72 h enzyme activity at pH 3 was no longer detectable. Such data and the results with the other isolates indicate that the time course of protease expression in the acid and the alkaline pH range may be different.

Furthermore our results (not shown in detail) on the rate of α-protein degradation and protease expression indicate that the different tempe forming *Rhizopus* isolates investigated may be devided into 4 groups with regard to the time required for digestion of α-protein grains. These groups are a) isolate Fi with 24 (± 2) h, b) the isolates Mala, CM, MS5, EN, and NRRL 2710 with 35 (± 2) h, c) the isolates Sama and MS1 with 42 (± 3) h, and d) isolate Sur with 135 (± 3,5) h. These data represent the average of 5 independent experiments per isolate. Furthermore all *Rhizopus* isolates investigated here expressed the alkaline protease activity.

In opposition to Fukumoto *et al.* (1967), who stated that *Rhizopus* possesses only one protease type, we had to assume that another enzyme is also expressed, and that this new protease appears to be necessary for dissolving and hydrolyzing soy-protein grains. The existence of a second protease type expressed by *Rhizopus* was later confirmed by Banerjee and Bhattacharyya (1993), who described an alkaline protease in the medium of a newly isolated *R. oryzae*. Matsushima *et al.* (1981) and Castro *et al.* (1991) came to the conclusion that the ability of the fungi to produce acidic, neutral and alkaline proteinases is correlated with the acidification or alkalination of the growth medium.

Purification, identification, and characterization of proteases

A four-step purification procedure (see Materials and Methods) for characterization of the *Rhizopus* proteases was established using protein from the culture filtrate of the isolate Fi grown 48 h on SRP medium. Chromatography on a Mono Q column separated the protease mixture in a pepstatin A sensitive protease, active only under acidic conditions (ACP) and a PMSF sensitive protease, mainly active at alkaline pH (ALP). From 600 ml culture filtrate with a protein content of 888 mg the proteases ACP and ALP were finally obtained with a total activity of 13.6 and 17.4 ΔE_{410}/min and a recovery of 25.2 and 16.3%. ACP was obtained with a specific activity of 9.7 ΔE_{410}/min * mg protein and ALP with 26 ΔE_{410}/min * mg protein. The apparent purification factor for enzyme ACP was 656-fold and for protein ALP a 300-fold enrichment was reached.

ACP was clearly identified as an aspartic protease by inhibition with pepstatin A (Table I). On the other hand ALP activity was not affected by either aspartic- or metallo-protease inhibitors (Table I). Typical cysteine proteinase inhibitors had little or no inhibitory effect on ALP, whereas PMSF and chymostatin caused 100 % inhibition, PMSF is known to inhibit serine proteases. Chymostatin is a specific inhibitor for chymotrypsin-like serine proteases, but it can also inhibit several

Table I. Inhibition of the acidic protease and the alkaline protease by different protease inhibitors.

Protease	Concentration/ assay	Activity (% of control)		
		ACP	ALP	
		pH 5.5	pH 7.5	pH 9.0
Metallo-proteases				
EDTA	5 mM	105%	108%	115%
Aspartic proteases				
Pepstatin A	0.1 mM	0%	100%	100%
Cysteine proteases				
Cystatin	0.1 mg/ml	100%	86–91%	100%
E-64	0.1 mg/ml	100%	70–75%	100%
Serine (and some cysteine) proteases				
PMSF	1 mM	100%	0%	0%
Chymostatin	1 mM	100%	0%	0%
Serine proteases				
Soybean Kunitz inhibitor	0.113 mg/ml	100%	95%	91%
Soybean Bowman-Birk Inhibitor	0.141 mg/ml	100%	91%	88%

Purified preparations of ACP (specific activity 9.7 ΔE_{410}/min * mg protein) and ALP (specific activity 26 ΔE_{410}/min * mg protein) were used for the inhibition studies in concentrations ranging from 5–38 μg protein/ml. For details see Materials and Methodes.

cysteine proteases (Umezawa, 1977). In essence, the inhibition data indicate that ALP seems to belong to the class of serine proteases.

The properties of ALP such as pH- and temperature optima as well as pH-stability (Table II), are very similar to those reported for subtilisin Carlsberg from *Bacillus licheniformis* (Aunstrup, 1980). Probably, *Rhizopus*-ALP like other microbial serine proteases belongs to the family of subtilisins. Another evidence for fungal serine proteases being members of the subtilisin family has been provided by Tatsumi *et al.* (1989) who found 44 % homology in the amino acid sequence of an alkaline 33 kD serine protease from *Aspergillus oryzae* with proteases from the subtilisin family.

Molecular weights determined under native conditions by gelfiltration and under denaturing conditions by SDS-PAGE were found to be ~35 kD for ACP and ~33 kD for ALP (Table II). Both proteases isolated from the culture filtrate did not bind to Con A.

Purified ACP and ALP could be separated into a considerable number of multiple forms. Using two independent methods, IEF and chromatofocusing, altogether 3 ACP forms were obtained (Table III). Their isoelectric points (IEPs) were determined to be 5.9, 5.0, and <4 (3.4 by IEF). Based on their IEPs the isoforms 5.9 and 5 appear to be the well-known *Rhizopus* pepsins (Tsuru *et al.*, 1970; Graham *et al.*, 1973). In contrast to Wang and Hesseltine (1970) both *Rhizopus* pepsins had, using soyprotein as substrate, identical pH optima with 2 peaks, one at about pH 3 and another one at pH 5.5. In the case of ALP at least 6 isoforms from isolate Fi were isolated by chromatofocusing (Table III), of which the two isoforms with IEP 7.1 and 6.5 were the most active ones. As an example for such studies on isoform separation, Fig. 2 shows one of the two chromatofocusing analyses with ALP in the pH range of 7–4. For determination of molecular weights the various isoforms were subjected to gelelectrophoresis; the data are presented in Table II. All ALP-isoforms were reproducibly found even after using the modified purification procedure. The isolation of isoforms with different IEPs (Table III) but identical molecular weights may be explained by the findings of Delany *et al.* (1987) and Takahashi (1988). These authors determined the amino acid sequences of the two *Rhizopus* pepsin isozymes IEP 5.9 and 5.0 and showed that 8 amino acids were not identical (Delany *et al.*, 1987). This difference led to a change in the charge of the protein but not in the molecular weight. Thus the existence of the 6 ALP-isoforms with identical molecular mass can be explained. Nevertheless it cannot be excluded that the ALP isoforms with a low IEP arise from high IEP-isoforms by molecular conversion. Ichishima *et al.* (1986), who described 5 multiple forms of a serine protease from *Aspergillus sojae* with identical size, discussed such a phenomenon. On

Table II. Properties of 2 acidic (ACP) and 2 alkaline (ALP) protease isoforms from *Rhizopus oryzae* isolate Fi after growth on soyprotein-raffinose-phytate medium using the soyprotein assay. Isoforms were obtained by chromatofocusing.

	ACP isozymes		ALP isozymes	
IEP	5.9	5.0	7.1	6.5
Molecular weight (kD)	~35	~35	~33	~33
Temperature optimum (°C)	55	55	55	55
pH optimum (at 55 °C)	~3 & ~5.5	~3 & ~5.5	~7	~7 & ~9
pH stability (at 4 °C, 4 h)	2.5–6.0	2.5–6.0	4–11	4–11

Table III. Acidic and alkaline protease isoforms of different *Rhizopus* strains: Fi (*R. oryzae*), Sur (*R. microsporus* var. *chinensis*), Mala (*R. oryzae*) and NRRL 2710 (*R. oligosporus*). The quantitative distribution of ACP and ALP activities on the isoforms is shown for isolate Fi.

Proteases	IEPs of ACP isoenzymes			IEPs of ALP isoenzymes							
No.	1	2	3	1	2	3	4	5	6	7	8
Fi / Activity	6.0–5.9 (32%)	5.1–4.9 (30%)	<4 (38%)	>9.6 (14.6%)	9.5 (6.8%)	8.5–8.4 (10.5%)	–	7.3–7.1 (24.9%)	6.6–6.5 (40.4%)	–	<4 (2.8%)
Sur	6.0–5.9	5.3–5.1	<4	–	–	–	–	–	–	–	–
Mala	6.1–5.9	4.9–4.8	<4	–	9.5	–	7.7	7.3–7.1	–	5.5	<4
NRRL 2710	6.0–5.9	5.2–5.1	<4	>9.6	–	–	–	–	–	–	<4

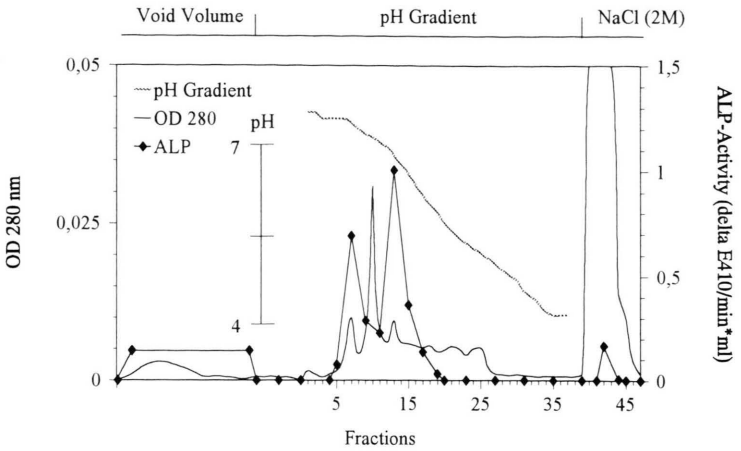

Fig. 2. Separation of ALP isoforms IEP 7.1, 6.5, and <4 by chromatofocusing in the pH range 7–4. The fraction size was 0.5 ml. Proteolytic activity in the eluates was detected at pH 9.0 using the azocasein assay. OD = optical density.

the other hand self incubation of ALP for 4 h (25 °C, pH 7.5) or repeated chromatofocusing did not lead to further isoforms.

For comparison of the different *Rhizopus* strains regarding their protease isoform patterns a three-step purification procedure (acetone precipitation, Phenylsuperose, Mono P) was established using protein from 48 h old culture filtrates. Although the isoform pattern was reproducibly found with each strain, the *Rhizopus* strains appear to differ in the number of ALP isozymes (Table III). Quite remarkably, *R. microsporus* var. *chinensis* (isolate Sur) totally failed to express ALP and *R. oligosporus* NRRL 2710 expressed only 2 ALP isoforms. The remaining two strains (Table III) were identical with regard to the isoforms with IEPs 9.5, 7.3–7.1 and <4, respectively. However, slight differences among these two strains were also found. Future studies will have to demonstrate whether different developmental stages of the fungi contribute to the number and IEPs of the various isoforms.

In vitro digestion of soyprotein

Degradation of commercial α-protein and salt-soluble soyprotein by both purified ACP and ALP at their respective pH optima occurred over the complete range of the soyproteins with equal intensity as demonstrated by protein gel electrophoresis of samples taken during a 48 h incubation period (data not shown). No preference for high molecular weight soyproteins in comparison to small proteins could be observed. Incubation controls (48 h, pH 9.0) without enzyme showed a poor degradation of soyprotein by alkaline hydrolysis only. However, digesting α-protein and salt-soluble protein by an ACP and ALP mixture at pH 6.5, which is the pH value determined in tempe extracts up to 15 h fermentation, revealed that the high molecular weight proteins were preferentially hydrolyzed. The total degradation of salt-soluble protein after 48 h in contrast to the α-protein preparation may be a question of the protein-protease ratio.

These data on soyprotein hydrolysis by fungal proteases ACP and ALP led to the assumption that the fermentation-caused pre-digestion of the soybean seed proteins may improve the digestibility of these proteins by mammalian proteases. However, an improved protein bio-availability of tempe samples prepared with our chosen isolates remains to be proven by animal tests.

Fungal proteases and soybean inhibitors

Among the soybean seed storage proteins the Kunitz inhibitor (KI) and the Bowman-Birk inhibitor (BBI) have received considerable attention (Winarno and Reddy, 1986). Therefore, these proteinase inhibitors have also been tested for their inhibitory activity on both ACP and ALP.

As expected ACP, an aspartic protease, was not inhibited by these two soybean serine proteinase inhibitors. Application of the soybean inhibitors according to the manufacturer's guide (inhibitor-

enzyme ratio: 1:3) failed to show any affect on ALP. Only with an increased inhibitor-enzyme ratio of 3:1 could some inhibition of ALP (Table I) be measured.

Incubating *Rhizopus* proteases with KI and BBI for a long period (48 h) using a very high inhibitor-protease ratio of 20:1 a significant (about 50%) degradation of KI and BBI was found (data not shown). For tempe fermentation only the BBI, which can inhibit trypsin and chymotrypsin simultaneously, is of special interest, because the KI will probably be destroyed during the cooking phase of the soybeans (Kunitz, 1947). As the *Rhizopus* proteases are not significantly inhibited by the soybean inhibitors these enzymes appear to be well suited for proteolytic digestion of the seed protein inhibitors during tempe fermentation.

In essence, our studies document that the *Rhizopus* proteases ACP and ALP are powerful enzymes for the proteolysis of two prominent protein preparations (α-protein and salt-soluble protein) of soybean seeds. Future studies will have to elucidate the protease pattern expressed by the *Rhizopus* isolates during soybean fermentation to yield tempe.

Acknowledgement

We thank Prof. Dr. H. J. Rehm, Institut für Mikrobiologie, Universität Münster for donating the *Rhizopus* isolates. This work was supported by the Bundesminister für Forschung und Technologie, Bonn, Germany and Fonds der Chemischen Industrie. M.-L. Heskamp acknowledges the receipt of a fellowship from the Konrad-Adenauer-Stiftung e.V.

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