Oryzasin as an Aspartic Proteinase Occurring in Rice Seeds: Purification, Characterization, and Application to Milk Clotting

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An aspartic proteinase in rice seeds (oryzasin) was purified by $(NH_4)_2SO_4$ fractionation, DEAE-celluose anion exchange chromatography, Sephadex G-100 gel filtration, Mono Q anion exchange chromatography, and pepstatin-affinity chromatography. SDS−PAGE showed the affinity-purified enzyme to have two molecular forms, 57 and 53 kDa, together with their probable autolysates appearing as two small bands at 35 and 25 kDa. Compared with the other three bands, the 57 kDa band reacted strongly on western blot analysis. The affinity-purified oryzasin pH optimum for hydrolysis is 3.0 and is completely inhibited by pepstatin but not affected by other proteinase inhibitors such as EDTA, leupeptin, PMSF, and E-64. The milk-clotting activity of oryzasin was investigated using the crude enzyme obtained by precipitation at 30% and 60% $(NH_4)_2SO_4$ saturation. The enzyme clotted a skim milk solution at pH 6.3, yielding the same κ-casein digest pattern as those of chymosin and pepsin producing a 12 kDa band.

Keywords: *Oryzasin; rice seed; aspartic proteinase; milk clotting*

INTRODUCTION

Enzymes belonging to the aspartic proteinase (AP) class (EC 3.4.23) deserve special note because of their involvement in physiologically important events including intracellular and extracellular protein catabolism. Chemically, each of the APs is characterized by the presence of two special aspartic acid residues constituting the active site (John, 1985) and by the loss of catalytic function upon treatment with pepstatin, azoacetylnorleucine methyl ester (DAN), 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP), etc., which specifically block the active site (Foltman, 1985). APs of animal and microbial origin have been extensively studied and their biochemical and physiological functions clarified. Pepsin and chymosin are representative digestive enzymes, each a monomeric protein of 35 kDa (Chen et al., 1975; Marciniszyn et al., 1975; Sepulveda et al., 1975; Foltman et al., 1979). Renin, a blood-pressure regulator purified from kidney, is comprised of a glycoprotein of 36−42 kDa (Inagami et al., 1985). Cathepsin D, on the other hand, a lysosomal enzyme is 40−45 kDa glycoprotein (Shewale et al., 1985) involved in intracellular protein catabolism (Nishimura et al., 1989; Barát et al., 1979). A similar AP occurs in yeast vacuoles, which is known as proteinase A and is a monomeric protein of 41 kDa (Dreyer et al., 1985).

Compared with the above proteins, plant APs are poorly documented. Recently, we obtained AP cDNA clones from ripening rice seeds shortly after flowering and investigated in detail one of the encoded proteins (Asakura et al., 1995a). This protein, named oryzasin 1, consists of 509 amino acid residues including signal peptide and pro-peptide sequences comprising 20 and 47 amino acid residues, respectively. An homology

search has shown that oryzasin 1 has a close structural similarity to HvAP, a barley seed AP (Runeberg-Roos et al., 1991), and cyprosin, a cardoon flower AP (Cordeiro et al., 1994), and rice aspartic proteinase (Hashimoto et al., 1992). These three well-defined APs of plant origin are each characterized by the presence of an approximately 100-amino acid insertion near the C-terminal region that has not been reported for APs of animal and microbial origin (Imai et al., 1983; Sogawa et al., 1983; Faust et al., 1985; Azuma et al., 1989; Harris et al., 1982; Woolford, et al., 1986).

There are various differences among the three plant APs, although they have high percentage identity to one another in terms of amino acid sequence. Structurally, HvAP is a heterodimer of 48 kDa, while cyprosin is a glycoprotein and occurs still as a heterodimer with a similar molecular size (49 kDa) (Heimgartner et al., 1990). Furthermore, HvAP has an optimum pH of 3.5−3.9 against hemoglobin as a substrate (Runeberg-Roos et al., 1991) while, interestingly, cyprosin exerts its greatest whole casein hydrolyzing activity at pH 5.1, the highest of all the optimum pH values observed for APs of plant origin (Heimgartner et al., 1990). In spite of these distinct features of plant APs, no information is available on their structure−activity relationships.

Some APs have milk-clotting activity and are used in cheese-making, such as bovine chymosin and *Mucor* rennin. Some plant APs also have similar activity, and cyprosin offers a good example (Faro et al., 1992). A proteinase occurring in a Sodom apple leaf extract may provide a similar example (Aworh and Nakai, 1986; Aworth and Muller, 1987). Oryzasin can be consistently and adequately obtained as a crude enzyme from a rice seed extract and, moreover, may be preferable from the aspect of food safety.

In the present study, we purified an AP from rice seeds and identified it as oryzasin 1, previously identified through molecular cloning (Asakura et al., 1995a). Here we report the characterization of this enzyme and also its possible utility in food processing.

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MATERIALS AND METHODS

Rice Seeds. Rice seed *Oryza sativa* L. japonica (cultivar Nipponbare), harvested at the farm of the Tokyo University Experimental Station, was used.

Purification Procedure. *Crude Enzyme Extract.* Shortly after harvest approximately 200 g of ripe rice seeds were finely ground in an electric coffee mill and suspended in 400 mL of 25 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl. The suspension was cooled in an ice bath and homogenized in a POLYTRON blender (Kinematica, Littau-Luzern, Switzerland) at its maximum speed for 10 min. The homogenate was centrifuged at 2000*g* for 5 min to obtain a supernatant, which was again centrifuged at 10000*g* for 20 min. The resulting supernatant was filtered through a cotton cloth to obtain the crude enzyme extract.

$(NH_4)_2SO_4$ Precipitation. $(NH_4)_2SO_4$ was added to the crude extract to a final concentration of 30%. The solution was allowed to stand for 3 h after which time the $(NH_4)_2SO_4$ concentration was increased to 60%. The resultant precipitate was collected and dialyzed against 25 mM phosphate buffer (pH 7.4).

Anion Exchange Chromatography. A column ($\varnothing$ 1.6 × 15 cm) packed with DE52 (Whatman) was equilibrated with 25 mM sodium phosphate buffer (pH 7.4). The $(NH_4)_2SO_4$ precipitated fraction was applied onto the column, washed with five column volumes of the same buffer, and eluted with 25 mM sodium phosphate buffer (pH 7.4) containing 0.3 M NaCl. Two column volumes of eluted fraction was collected and concentrated to about 1 mL by ultrafiltration through a Diaflo PM-10 membrane (Amicon, Beverly, MA).

Gel Filtration Chromatography. The concentrated solution was applied onto a Sephadex G-100 (Pharmacia LKB Biochemistry, Uppsala Sweden) column ($\varnothing$ 1.8 × 85 cm) and eluted with 25 mM sodium phosphate buffer containing 0.15 M NaCl (pH 7.4). The eluted fractions, 4 mL each, were assayed for protease activity as described later. Active fractions were pooled and dialyzed against 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl.

FPLC Ion Exchange Chromatography. A Mono Q column HR5/5 (Pharmacia LKB Biochemistry) was used. The column was equilibrated with 20 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl. Proteins were eluted by increasing the NaCl concentration in a stepwise fashion.

Pepstatin Affinity Chromatography. A pepstatin-Sepharose 4B affinity gel was prepared by coupling pepstatin A (Peptide Institute, INC., Osaka, Japan) to EAH-Sepharose 4B (Pharmacia LKB Biochemistry). Pepstatin A was dissolved in 75% MeOH. EAH-Sepharose 4B was suspended in 75% MeOH before coupling, a 12 mM pepstatin solution was added to the EAH-Sepharose 4B, and fresh EDC [*N*-ethyl-*N*-(3'-(dimethylamino)propyl)carbodiimide hydrochloride] was added to a final concentration of 0.1 M. The reaction solution was mixed slowly upside down for 24 h at room temperature with the pH of solution kept between 4.5 and 6.0. After the reaction, noncoupled pepstatin was removed by washing with 75% MeOH. Five milliliters of pepstatin Sepharose 4B was packed into a column and buffered with 0.1 M acetate buffer (pH 4.0) containing 1 M NaCl and 0.5% Brij (loading buffer). Samples were applied to the pepstatin affinity column, and the column was washed with loading buffer until the eluted fractions showed no more absorbance at 280 nm. The column was eluted with 0.1 M Tris-HCl (pH 8.2) containing 1 M NaCl and 0.1% Brij (elution buffer), aliquots (1 mL) were collected, and their protein concentration and protease activity were measured. All purification procedures were carried out at 4 °C.

Assay of Protease Activity. A 10–50 μL portion of enzyme solution was added to 50 μL of 1% acid-denatured hemoglobin solution. The mixture was incubated at 37 °C, pH 3.3, for 60 min, and the reaction was terminated by the addition of 100 μL of 0.4 M TCA. After centrifugation, the absorbance of the TCA-soluble fraction was measured at 280 nm. One unit of proteolytic activity was the amount necessary to produce an increase in absorbance of 0.01 under the conditions above.
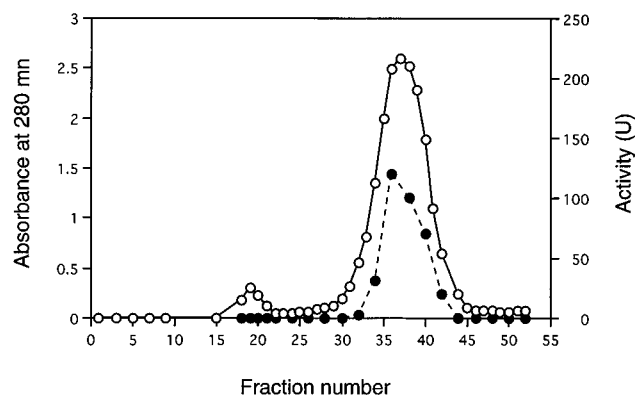

Figure 1. Gel filtration on Sephadex G-100 of the enzyme fraction obtained from DEAE-cellulose chromatography. About 12 000 units of enzyme were applied to a column, $\varnothing$ 1.8 × 85 cm, of Shephadex G-100 equilibrated with 25 mM sodium phosphate buffer containing 0.15 M NaCl (pH 7.4), and 4 mL fractions were collected. Solid and dotted lines show protein and proteolytic activity, respectively.

Measurement of Protein Concentration. Protein concentration was measured using a BCA kit (BCA protein assay reagent, Pierce, Rockford, IL) with bovine serum albumin as the standard.

Milk-Clotting Assay. Enzyme solution containing about 0.3 mg protein was added to 0.2 mL of 3% (w/v) skim milk reaction mixture dissolved in 25 mM sodium phosphate buffer (pH 6.3), 20 mM $CaCl_2$. The reaction tubes were inclined and incubated at 37 °C for 120 min.

κ**-Casein Digestion.** Each of 0.6 μg of oryzasin, 1.6 μg of chymosin (Sigma, St. Louis, MO, U.S.A.), and 1.6 μg of pepsin (Sigma) was mixed with 8 μL of 100 mM glycine hydrochloride buffer (pH 2.0), 100 mM acetate buffer (pH 3.0) or 100 mM sodium phosphate buffer (pH 6.3) solution containing 80 μg of κ-casein. The mixtures were incubated at 37 °C for 60 min and subjected to SDS−PAGE.

SDS−PAGE. Electrophoresis was performed on 10% or 15% polyacrylamide gels in the presence of 0.1% SDS according to the method of Laemmli (1970).

Antibody Preparation. The oryzasin 1 C-terminal region protein (Met[212]−Ala[509]; Asakura et al., 1995a) was expressed in *Escherichia coli* and purified by polyacrylamide gel elecrtrophoresis. About 100 μg of protein was extracted from the gel, mixed with Freund's complete adjuvant, and injected into a male rabbit. Boosters of 100 μg of protein were administered five times at 2-week intervals. The antiserum obtained was stored at −80 °C until used.

Western Blot Analysis. Polyvinylidene difluoride membranes were obtained from Millipore, and the anti-rabbit IgG alkali phosphatase conjugate was purchased from Sigma. Samples were electrophoresed as described above and transferred into polyvinylidene difluoride membranes according to Watabe et al. (1993).

RESULTS

Purification of Oryzasin. The crude extract resulting from 30−60% $(NH_4)_2SO_4$ precipitation was applied to a DEAE-cellulose column, and the oryzasin was collected in the 0−0.3 M NaCl eluted fraction. The enzymatically active fraction was eluted, concentrated to 1 mL by ultrafiltration, and applied to a Sephadex G-100 gel filtration column from which the activity eluted in fractions 33−42 (Figure 1). This peak was then subjected to Mono Q column chromatography to obtain three active fractions named P1, P2, and P3, eluting at NaCl concentrations of 0.1−0.15, 0.15−0.2, and 0.2−0.3 M, respectively (Figure 2). The fractions for each peak were pooled and concentrated by ultrafiltration. Analysis of these fractions gave total protein, total protease activity, and specific activity data as
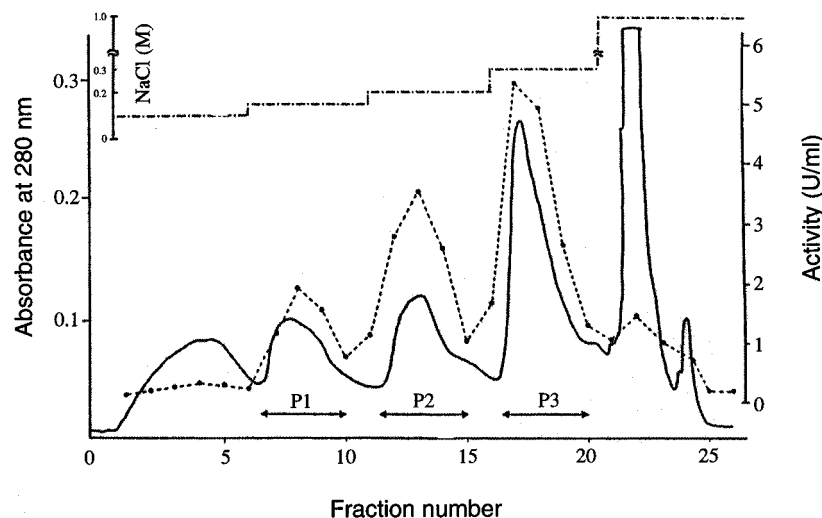
Figure 2. Mono Q chromatography of the enzyme fraction obtained from Sephadex G-100 gel filtration. A Mono Q column (HR 5/5) was equilibrated with 20 mM Tris-HCl buffer containing 0.1 M NaCl (pH 8.0). The elution was carried out by increasing the NaCl concentration stepwise from 0.1 to 1.0 M. The flow rate was 0.5 mL per min, and fractions of 1 mL each were collected. The fractions containing proteolytic activity were designated P1, P2, and P3. Solid, dotted, and broken lines show protein, activity, and NaCl concentration, respectively.
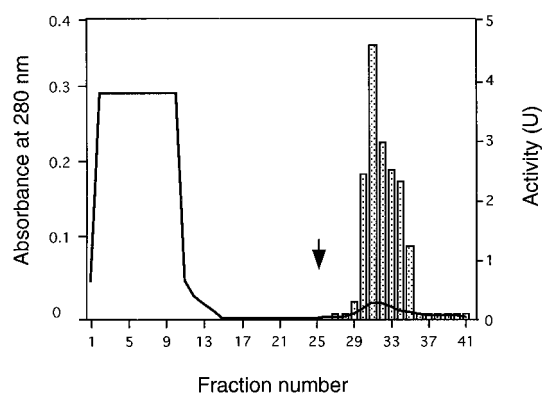

Figure 3. Pepstatin-Sepharose 4B affinity chromatography of the Mono Q P2 fraction. The concentrated P2 fraction was applied to a column of 5 mL pepstatin-Sepharose 4B equilibrated with 0.1 M sodium acetate buffer (pH 4.0) containing 0.1 M NaCl and 0.5% Brij 35. The arrow indicates the buffer change to 0.1 M Tris-HCl buffer (pH 8.2) containing with 1 M NaCl, 0.1% Brij 35. Fractions of 1 mL each were collected. The solid line represents protein, and boxes indicate proteolytic activity.

Figure 4. SDS−PAGE and western blot analysis of affinity-purified oryzasin. (A) 10% SDS polyacrylamide gel. (B) Western blot analysis. The marker positions are shown on the left of A and to the right of B. Arrows indicate the resulting bands.

follows: 2.2 mg, 503 units, and 229 units/mg for P1; 2.8 mg, 877 units, and 313 units/mg for P2; and 6.4 mg, 1195 units, and 187 units/mg for P3.

Total recovery at this stage was 11.4 mg of total protein and 2575 units of total activity. This indicates that the enzyme was recovered with the yield of 31.2% from the previous step. For further purification, we thought it reasonable to use P2, which had the highest specific activity (313 units/mg), accounting for 34% of the total.

When P2 was applied to a pepstatin-conjugated column, strong AP activity eluted in fractions 29−35 when the column was washed with elution buffer (Figure 3). This affinity chromatography made it possible to increase the specific activity 10-fold from the previous stage and about 50-fold from the initial crude extract (Table 1).

The 30−60% $(NH_4)_2SO_4$-precipitated fraction from the crude enzyme extract and the Mono Q fractions (P1, P2, and P3) were subjected to SDS−PAGE. Western blot analysis using an anti-oryzasin 1 antibody revealed several positive bands. Among them, only two bands,
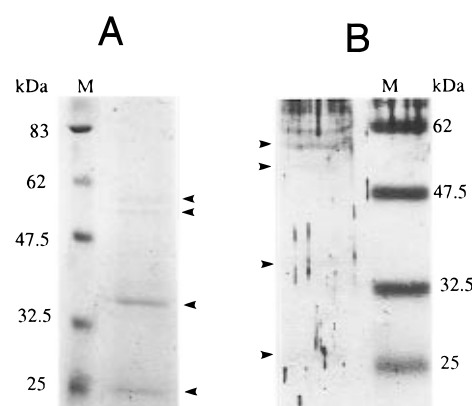
57 and 35 kDa, were commonly observed in all lanes other than the marker lane (data not shown). The P1, P2, and P3 fractions were all found to have AP activity and their western analyses also showed the 57 and 35 kDa bands. SDS−PAGE of affinity-purified oryzasin gave weak 57 and 53 kDa bands together with strong 35 and 25 kDa bands. Western blot analysis of the same fraction showed, in contrast, a strong 57 kDa band compared to the 53 kDa band, while the other two bands, 35 and 25 kDa, were weak (Figure 4). The western blot analysis gave the result that 57 and 53 kDa bands weakly stained by the Coomassie Brilliant Blue treatment cross-reacted more strongly than 35 and 25 kDa bands did.

Effects of Various Inhibitors. Affinity-purified oryzasin activity was inhibited completely by pepstatin but was not affected by other inhibitors (Table 2).

Optimum pH for Oryzasin Activity. As Figure 5 shows, oryzasin has its maximum activity around pH 3 and gradually loses activity as the medium pH exceeds pH 4. For casein, the activity gradually decreases with increasing pH.

Temperature Dependency of Oryzasin Activity. The hemoglobin hydrolyzing activity of oryzasin was measured in the temperature range 23−57 °C. As

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Table 1. Recovery of Activity and Yield of Rice Aspartic Proteinase at Various Purification Steps

purification step	total protein (mg)	total activity (units)	specific activity (units/mg)	yield (%)
1. crude extract	839	50 700	60.4	100
2. $(NH_4)_2SO_4$	147	14 070	95.7	27.8
3. DEAE-cellulose	74	12 000	162	23.7
4. Sephadex G-100	37	8 150	220	16.1
5. Mono Q	2.8	877	313	1.7
6. pepstatin-Sepharose 4B	0.047	138	2 936	0.27

Table 2. Effect of Various Reagents on the Activity of Rice Aspartic Proteinase[a]

inhibitor	concentration (mM)	relative activity (%)
none	0	100
pepstatin	0.1	0
EDTA	1	100
leupeptin	0.01	104
E-64	0.1	97
PMSF	0.5	97

[a] Reactions were carried out with acid-denatured hemoglobin as a substrate in 0.1 M acetate buffer (pH 3.3) at 37 °C for 60 min.

Figure 7. Coagulation of skim milk with crude enzyme solution from rice seeds. The reaction mixture contained 3% skim milk, 20 mM $CaCl_2$, 25 mM phosphate buffer (pH 6.3), and 0.3 mg of the 30−60% $(NH_4)_2SO_4$ precipitated fraction from rice extract. Lane 1, without enzyme solution; lane 2, without inhibitor; lane 3, 0.1 mM pepstatin; lane 4, 1 mM EDTA; lane 5, 0.01 mM leupeptin; lane 6, 0.05 mM PMSF. In each case, incubation was carried out at 37 °C for 120 min.
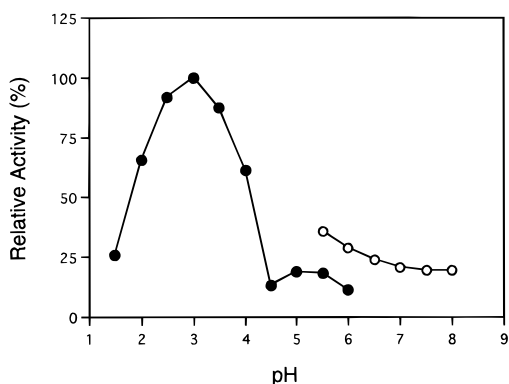

Figure 5. pH dependency of the hemoglobin and casein digesting activity of oryzasin. Reaction mixtures containing 10 units of affinity-purified enzyme and 1% acid-denatured hemoglobin or 1% casein at various pH were incubated at 37 °C for 60 min. Filled and open circles show hemoglobin and casein, respectively.
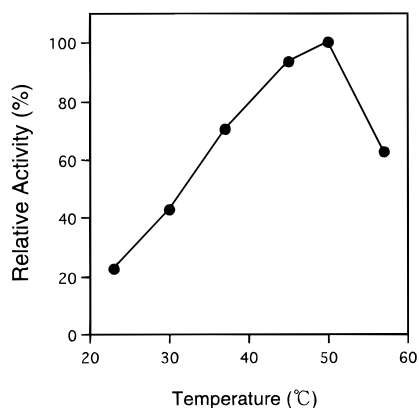

Figure 6. Effect of temperature on activity of oryzasin. Affinity-purified enzyme samples, 10 units each, were assayed in 0.1 M acetate buffer (pH 3.3) containing 1% hemoglobin at incubation temperatures ranging from 23 to 57 °C.

Figure 6 shows, the activity increases gradually as the temperature rises. Above 50 °C, the activity decreases markedly.

Milk-Clotting Activity of Oryzasin. The crude enzyme preparation in the 30−60% $(NH_4)_2SO_4$ precipitate (Table 1) was assayed for the ability to clot skim milk. The crude enzyme preparation was dissolved in buffer at a protein concentration of 0.15%, and the solution was added to 3% skim milk at pH 6.3. The
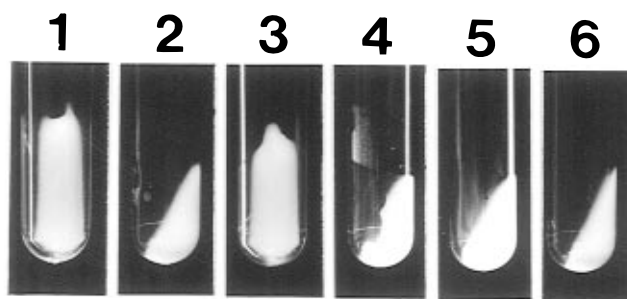
mixture in the test tube was then allowed to stand at 37 °C for 2 h, and the resultant coagulation was observed. The presence or absence of milk-clotting activity was judged by observing whether or not the incubation mixture showed sedimented coagulum formation. A 25 mM phosphate buffer (pH 7.4) was mixed with skim milk to confirm that the mixture showed no coagulation (Figure 7, lane 1). In the presence of crude enzyme, however, a completely gelled state resulted (lane 2). When pepstatin was added prior to incubation, the resulting incubation mixture did not gel (lane 3). The addition of EDTA, leupeptin, or PMSF did not prevent gel formation (lanes 4−6, respectively).

Specific Proteolysis of κ-Casein by Oryzasin. It is well-known that the specific hydrolysis of κ-casein in milk triggers its clotting leading to the formation of a cheese-like gel. The cleavage point in this hydrolysis has been determined to be the peptide bond between Phe[105] and Met[106] of κ-casein. Although there are several κ-casein variants depending on the size of the sugar chains attached, the main molecular species is 28 kDa as confirmed in our SDS−PAGE experiments (Figure 8, lane 1). When a κ-casein solution (1%) was treated with oryzasin at pH 6.3, the original band, 28 kDa, disappeared with the formation of a new 12 kDa band (Figure 8, lane 2). Treatment of κ-casein with either pepsin or chymosin instead of oryzasin, also at pH 6.3, gave almost the same results (lanes 4 and 6). However, when κ-casein was treated with oryzasin at its optimum pH (pH 3), no limited proteolysis occurred and a variety of lower molecular weight peptides were formed (lane 3). These results indicate that around pH 6, oryzasin induces limited κ-casein proteolysis and could be useful as a milk-clotting enzyme for making a cheese-like gel.

DISCUSSION

Purified oryzasin is completely inhibited by pepstatin, not by other protease inhibitors. This results suggests that oryzasin is a pepstatin-sensitive AP. Western blot
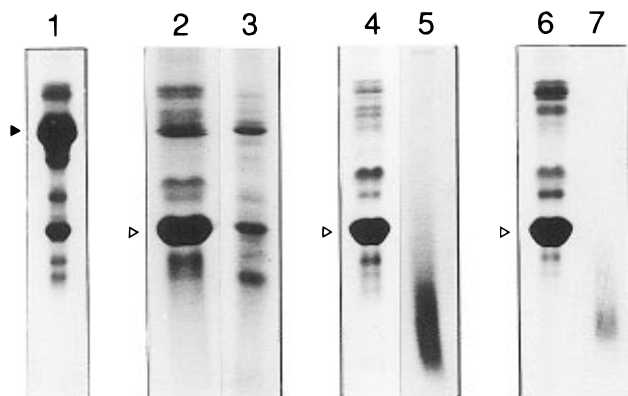
Figure 8. Proteolysis of κ-casein by oryzasin and other milk clotting enzymes. Lane 1, intact κ-casein; lane 2, 0.6 μg of oryzasin (pH 6.3); lane 3, 0.6 μg of oryzasin (pH 3.0); lane 4, 1.6 μg of chymosin (pH 6.3); lane 5, 1.6 μg of chymosin (pH 3.0); lane 6, 1.6 μg of pepsin (pH 6.3); lane 7, 1.6 μg of pepsin (pH 2.0). The filled arrow indicates 28 kDa intact κ-casein while the open arrows indicate 12 kDa products.

analysis showed that the affinity-purified oryzasin preparation cross-reacts with an anti-oryzasin 1 antibody, indicating that the preparation is either oryzasin 1 itself or a complex of structurally related enzyme species. Our preceding study using RT-PCR revealed several AP species in rice seeds (Asakura et al., 1995b). The same is true for the present study in which several AP peaks appeared when chromatography on Mono Q was carried out (Figure 2). Thus, the occurrence of plural oryzasin species is confirmed at the protein as well as the DNA level. Doi et al. (1980a,b) reported that two types of APs are existing in rice seeds.

Affinity-purified oryzasin appears to be a monomer since a single band, although weak, appeared at 57 kDa (Figure 4). This band crosses strongly with anti-oryzasin 1 antibody (Figure 4). The molecular mass estimation by cDNA cloning of oryzasin gave a value of 52 kDa for the *pro* form and 48 kDa for the mature form (Asakura et al., 1995a). There is thus a discrepancy of 5−9 kDa. Oryzasin is probably a glycoprotein since it has two potential glycosylation sites, Asn-His-Thr[254] and Asn-Lys-Thr[400] (Asakura et al., 1995a). Heimgartner et al. (1990) have reported that cyprosin is also a glycoprotein glycosylated at Asn[400] and Asn[447] (oryzasin numbering).

SDS−PAGE of purified oryzasin gave three bands other than the 57 kDa band. Among them, the 53 kDa band may result from the processing of the 57 kDa band. On the other hand, Coomassie Brilliant Blue staining showed two strong lower molecular weight bands, 35 and 25 kDa, which may arise from heterodimers produced by autodigestion. A similar autodigestive process has been reported for HvAP, a barley AP. In our case, however, it remains to be studied whether these two lower molecular weight species occur in rice seeds *per se* or are produced as artifacts during affinity chromatography under acidic conditions that can favor autodigestion. In any case, our study is the first to demonstrate the occurrence of oryzasin as a 57 kDa monomer, which has not been detected in barley.

Oryzasin is highly homologous (73% similarity) to cyprosin, an AP occurring in the flower of *Cynara cardunculas* and used for cheese-making. Actually, oryzasin is able to clot milk (Figure 7). Although there are various proteinases in rice seeds, the use of inhibitors showed that only oryzasin clots milk at neutral pH. In general, milk-clotting enzymes first attack κ-casein

that stabilizes the micelle structure in the natural milk system. Cyprosin is known to specifically attack κ-casein at the peptide bond between Phe[105] and Met[106] without further proteolysis at other peptide bonds (Macedo et al., 1993). Oryzasin was found to digest κ-casein in a restricted fashion with the formation of a digestion product with a molecular mass of 12 kDa, comparable to the size of products formed from κ-casein by digestion with pepsin and chymosin (Figure 8). Oryzasin is thus a new milk-clotting enzyme that may prove useful for cheese-making since a stable supply can be obtained.

ABBREVIATIONS USED

AP, aspartic proteinase; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; DAN, azoacetylnorleucine methyl ester; PMSF, phenylmethanesulfonyl fluoride; HvAP, barley grain aspartic proteinase.

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