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# Distribution of Chitinases in Rice (*Oryza sativa L*) Seed and Characterization of a Hull-Specific Chitinase

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**The uneven distribution of acidic and basic chitinases in different parts of rice seed, and also the characterization of hull-specific chitinase, are reported here. After extraction of chitinases from polished rice, bran, and rice hulls, the chitinases were separated into acidic and basic fractions, according to their behavior on an anion exchanger column. Both fractions from different parts of rice seed showed characteristic activity bands on SDS-PAGE that contained 0.01% glycol chitin. The basic chitinases from rice hulls were further purified using chitin affinity chromatography. The chitinase, specific to rice hulls (RHBC), was 88-fold purified with a 1.3% yield. RHBC has an apparent molecular weight of 22.2 kDa on SDS-PAGE. The optimal pH and temperature were 4.0 and 35°C, respectively. With [$^3$H]chitin as a substrate, RHBC has $V_{max}$ of 13.51 mg/mg protein/hr and $K_m$ of 1.36 mg/ml. This enzyme was an endochitinase devoid of β-1,3-glucanase, lysozyme, and chitosanase activities.**

**Keywords:** Rice seed, Chitinase, Distribution, Hull-specific, Purification

## Introduction

There is increasing evidence that plant chitinases play important roles, not only in defending plants (Boller, 1988), but also in regulating plant development (De Jong *et al.*, 1992) and signal transduction (Roche *et al.*, 1991). Since few seeds that germinate in soil are infected with pathogens, more attention is being focused on the biological functions of chitinases in seeds as a way to increase plant production. Most of the studies on chitinase evolution, and the physiological conditions that elicit the production of rice chitinases, are carried out in rice cell culture (Kim *et al.*, 1994, Xu *et al.*, 1996), or in germinating seeds or young seedlings (Nishizawa and Hibi, 1991, Zhu and Lamb, 1991, Nishizawa *et al.*, 1993, Kim *et al.*, 1998). However, there is little information on their distribution or nature in rice grains themselves, although the total chitinolytic activity in rice grains has been reported (Hirano *et al.*, 1989). Recently, we have reported that there is a difference in the activity distribution of endochitinases, β-*N*-acetylglucosaminidases, and β-1,3-glucanase between the hulls and bran of rice (Han *et al.*, 1998).

In this paper, we report that different molecular weight chitinases have characteristic distributions in polished rice, bran, and rice hulls. Furthermore, we purified a hull-specific chitinase and studied its enzymatic properties.

## Materials and Methods

**Materials** Whole grains of rice (variety Akibari) were purchased in the market immediately after harvest, and stored at a constant temperature and humidity. The regenerated chitin was prepared with the method of Molano *et al.* (1977). The packed anion exchanger column, Resource-Q (6 ml) and Source-Q, were purchased from Amersham-Pharmacia. [$^3$H]-acetic anhydride (500 mCi/mmol) was purchased from Amersham (England). All of the other chemicals were obtained from Merck (Germany) or Sigma (St. Louis, USA) and were of analytical grade.

**Extraction and purification** Polished rice and hulls were further crushed and then passed through a 20-mesh sieve. The polished rice, bran, and hulls were extracted using the previously described method (Han *et al.*, 1998). The protein extracts were centrifuged at 3,000 g for 10 min, then at 20,000 *g* for 20 min. After adjusting the pH to 7.5, 6 mg of protein was loaded onto a prepackaged Resource-Q anion exchange column (6 ml), which was previously equilibrated with a 0.02 M Tris-HCl buffer (pH 7.5). The column was thoroughly washed with a loading buffer before gradient elution with 0.0 M to 0.5 M NaCl. By this step the acidic and basic fractions were separated for SDS-PAGE. For the purification of the hull-specific chitinase, the crude extract was concentrated 25 times with an ultrafiltration membrane (MW cut-off: 10,000). It was dialyzed against 0.02 M Tris-HCl buffer (pH 7.5) before the anion exchanger chromatography. A Source-Q anion exchange column

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(2.6 × 15 cm) was used instead of the prepackaged column of Resource-Q. The flow rate was 8 ml/min. One fraction contained 20 ml. The unbound fraction was dialyzed against 0.02 M sodium bicarbonate (pH 8.4), and applied to a regenerated chitin column (1.6 × 15 cm) that was previously swollen in the same buffer. The column was successively eluted with 100 ml of the initial buffer, 0.02 M acetate buffer (pH 5.5), and 0.02 M acetic acid (pH 3.2). The flow rate was 3 ml/min and fractions of 3 ml were collected.

**Electrophoresis and detection of enzyme activity** SDS-PAGE was performed using the method of Laemmli (1970) in the presence of 0.01% glycol chitin. The bands of enzyme activity were detected with Calcofluor White M2R under UV light after treating the gel with Triton X-100 (Trudel and Asselin, 1989).

**Enzyme assays** Chitinase activity was measured using [$^3$H]chitin, as previously described (Molano *et al.*, 1977). [$^3$H]Chitin was suspended in the extraction buffer (1 mg/ml, w/v), and 100 µl was taken under continuous agitation. Five hundred and eighty eight dpm were equivalent to 1 µg of dry chitin. The radioactivity was quantified with a β-scintillation counter (Tricarb 1600, Packard). For a kinetic study 35,000 dpm to 1,570,000 dpm of [$^3$H]chitin were added to 100 µl of the purified enzyme solution (1.34 µg/ml of a 0.05 M citrate-phosphate buffer, pH 4.0). The final volume was adjusted to 1.5 ml. After a 45 min incubation at 35°C at pH 4.0, boiling at 100°C for 10 min stopped the reaction. Since [$^3$H]chitin was used as a substrate, $K_m$ and $V_{max}$ were expressed mg/ml and mg/mg protein/hr instead of mM (Han *et al.*, 2000). When oligomers of *N*-acetylglucosamine (GlcNAc) were used as substrate, 1 mg of oligomers was added to 1 ml of the citrate-phosphate buffer (0.05 M, pH 4.0). Aliquots of 100 µl were removed at appropriate time intervals. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 µmol of monomers per minute at 35°C. β-*N*-acetylglucosaminidase assays were carried out with *p*-nitrophenyl-β-*N*-acetylglucosaminide in a citrate-phosphate buffer (0.05 M, pH 4.5) (Ohtakara, 1989). Lysozyme activity was measured according to the method described by Martin (1991). β-1,3-Glucanase activity was checked using laminarin as a substrate, according to the method described by Fink *et al.* (1988).

**Analysis of the reaction products by TLC** The reaction mixture was applied on a pre-coated silica gel G255 plate and developed twice with a solvent system of isoamyl-alcohol : ethanol : water : $NH_4OH$ (50 : 60 : 36 : 1, v/v). After spraying a mixture of 1% *o*-aminophenol : $H_3PO_4$ : water (1 : 2 : 1, v/v), the plate was heated at 110°C for 1 h to obtain color development products.

**Determination of $NH_2$-terminal amino acid sequence** The purified enzyme was blotted on a PVDF membrane. Automated Edman degradation was performed with protein/peptide sequencer 471A (Applied Biosystem, USA), according to the method described by the manufacturer.

## Results and Discussion

**Distribution of basic and acidic chitinase activity in polished rice, bran, and hulls of rice seed** A prepackaged Resource-Q column (6 ml) was used to separate the crude preparations into acidic and basic chitinases. The effluent fractions contained basic proteins, while the eluant fractions contained acidic proteins (Fig. 1A).

As seen in Fig. 1B, four major bands of chitinase activity were detected in the gel by staining the gel with Calcofluor White M2R: HMC1 (43.5 kDa), HMC2 (32 kDa), LMC1 (24.5 kDa), and LMC2 (22 kDa). In polished rice, HMC2 was the main chitinase(s). It was primarily localized in the basic fraction. In rice bran, however, HMC2 and LMC1 were the major isozymes. LMC1 had a higher activity than HMC2 in the basic fraction. In rice hulls LMC1 and LMC2 were the major molecular species of chitinases, although HMC1 was the most abundant. HMC2 had relatively a lower activity than the activity that was found in both polished rice and rice bran. In addition, LMC2, which was uniquely observed in rice hulls, was basic in nature. This unique LMC2 was the objective of further characterization. Since hulls are the first part of the rice seed formed, and it is the first mechanical and biochemical barrier against possible invaders, we expect the hulls to contain more molecular species of chitinase, as well as the highest chitinase activity. Tissue-specific differences in the patterns of chitinases transcripts accumulate in the root, but the levels found in stems and leaves are barely detectable (Zhu and Lamb, 1991). The unique presence of LMC2 in rice hulls, and the unequal distribution of the 4 major molecular species of chitinases in rice seed, has not been reported previously. The reported molecular weights of chitinases, isolated from rice seedlings (Kim *et al.*, 1998), range from 20 kDa to 27.6 kDa. In contrast, chitinases, obtained from rice cell cultures or deduced from cDNA (Pan *et al.*, 1996), have molecular weights between 40.5 kDa and 52.5 kDa. In rice suspension cells, a basic chitinase with a molecular weight of 33.1 kDa was identified (Xu *et al.*, 1996). This range of molecular weights agrees with that found in the three parts of rice seed. This suggests that the seeds containing chitinase isozymes are found in other tissues, or under different physiological conditions.

**Purification of the hull-specific chitinase** As seen in Fig. 2A, one unbound fraction (QF1) and 2 acidic fractions (QF2 and QF3) were obtained from rice hulls. QF1, containing hull-specific chitinase, represented 34% of the total activity and 9% of the total protein. When the unbound fraction QF1 was chromatographed on the regenerated chitin column using step-wise elution of different buffers, more than 60% of the activity was found in the poorly retained fraction (AF1), a pH 5.5 eluted fraction (AF2), and two pH 3.3 eluted fractions (AF3 and AF4) followed (Fig. 2B). While AF1 showed no consistent activity profile, the rest of the fractions were highly reproducible. Several reasons for the chromatographic inconsistency may be plausible. [1] It may be partly due to the packing of the column and degradation of the affinity matrix during the operation, even though the procedure was carried out at 4°C. [2] It may be caused by the presence of different

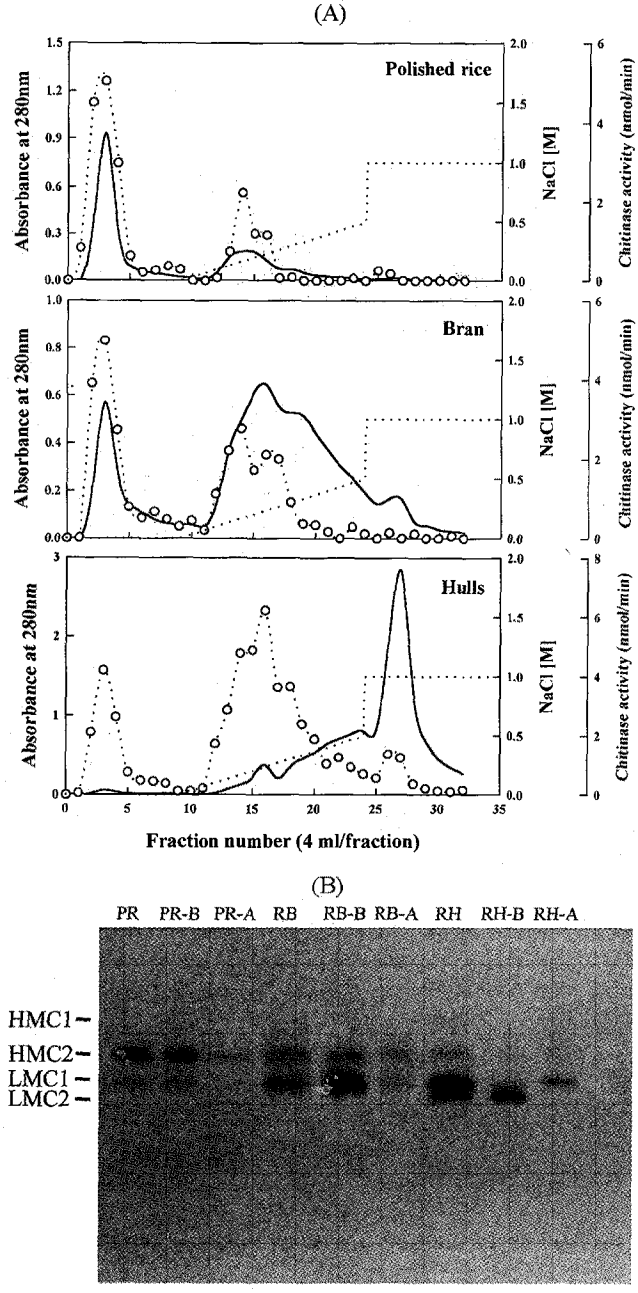


**Fig. 1.** Separation of acidic and basic chitinases and their activity bands from polished rice, bran, and hulls. (A) Anion exchanger chromatogram of polished rice, bran, and hulls. The column (6 mL) was prepackaged with Resource-Q. ——, Absorbance at 280 nm; --○--○--, Chitinase activity; - - - - -, NaCl gradient. (B) Chitinase activity bands of acidic and basic fractions obtained from polished rice, bran, and hulls of rice seed. Equal amount of proteins was applied to each lane. SDS-PAGE was carried out in the presence of 7.5% glycol chitin. The enzyme activity was detected with Calcofluor White M2R under UV. PR: Total extract of polished rice, PR-B: Basic fraction of PR, PR-A: Acidic fraction of PR, RB: Total extract of rice bran, RB-B: Basic fraction of RB, RB-A: Acidic fraction of RB, RH: Total extract of rice hulls, RH-B: Basic fraction of RH, RH-A: Acidic fraction of RH.

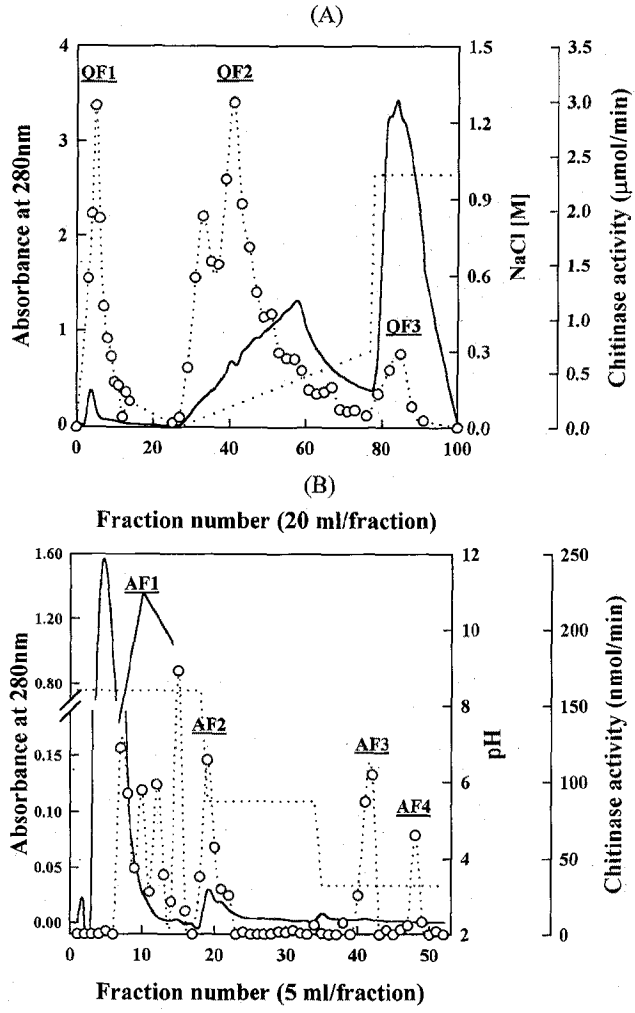


**Fig. 2.** Purification of a hull-specific chitinase (RHBC) from rice hulls by different chromatographic methods. (A) Anion exchanger chromatography on Source-Q (1.6 × 15 cm). (B) Chitin affinity chromatography of QF1 from Source-Q. ——, Absorbance at 280 nm; --○--○--, Chitinase activity; - - - - -, pH gradient.

chitinase isozymes, whose affinity toward chitin was relatively lower due to a deficiency in the cystein-rich domain (Collinge *et al.*, 1993). This AF2 fraction had a much lower chitin-binding affinity than a chitinase of pumpkin leaves, which could be eluted with 20 mM NaOH (Lee *et al.*, 1999).

As seen in Figs. 3A and. 3B, AF2 showed a single band that corresponded to the molecular weight of 22.2 kDa (Fig. 3A). It coincided nicely with the activity band that was previously shown in Fig. 1B (LMC2). This basic chitinase, specifically present in rice hulls (RHBC), was purified 88 times with a 1.3% yield of the enzyme activity (Table 1). Recently a rice-husk-specific chitinase with a molecular weight of 21.3 kDa, based on the amino acid sequence, was reported with no information about enzymatic properties (Nakazaki *et al.*, 1997). We obtained 15 sequences of $NH_2$-

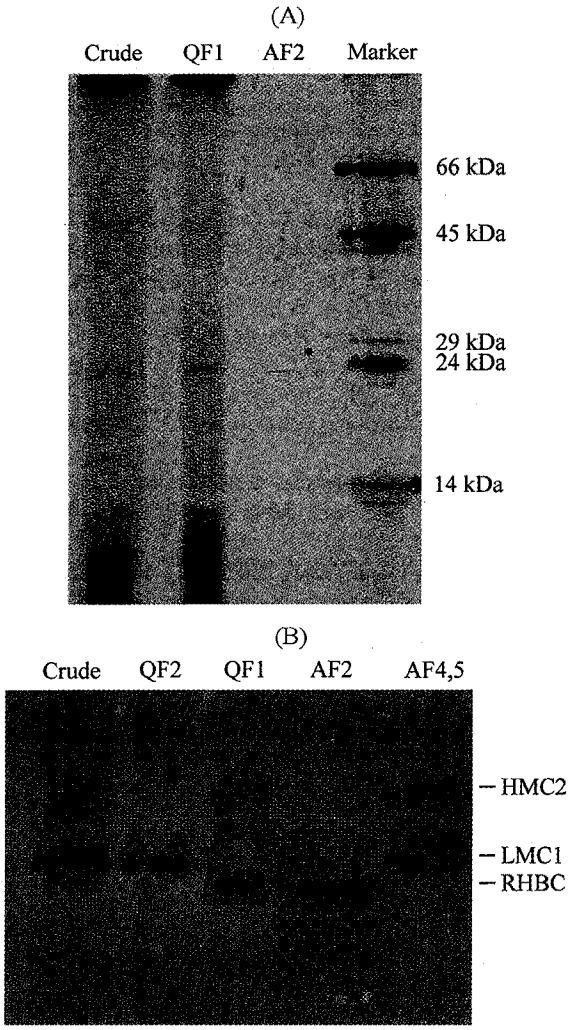


**Fig. 3.** SDS-PAGE pattern of the purified RHBC. (A) Protein staining with silver nitrate. (B) Activity band staining with Calcofluor White M2R

terminal of RHBC: *N*-Ala-Asn-Val-Ala-Asp-X-X-Tyr-X-X-Ala-Ile-Phe-His-*C* (X is either cystein or modified amino acid). When we compared this sequence with that of the rice-husk-specific chitinase (Nakazki *et al.*, 1997), there was only a 27% homology. However, RHBC shared 40%, 47%, and 40% homology with Rcht4 from rice (Park, 1999), chit A and chit B from maize (Huyuh *et al.*, 1992), respectively. This result may suggest that RHBC is a novel chitinase from rice bran.

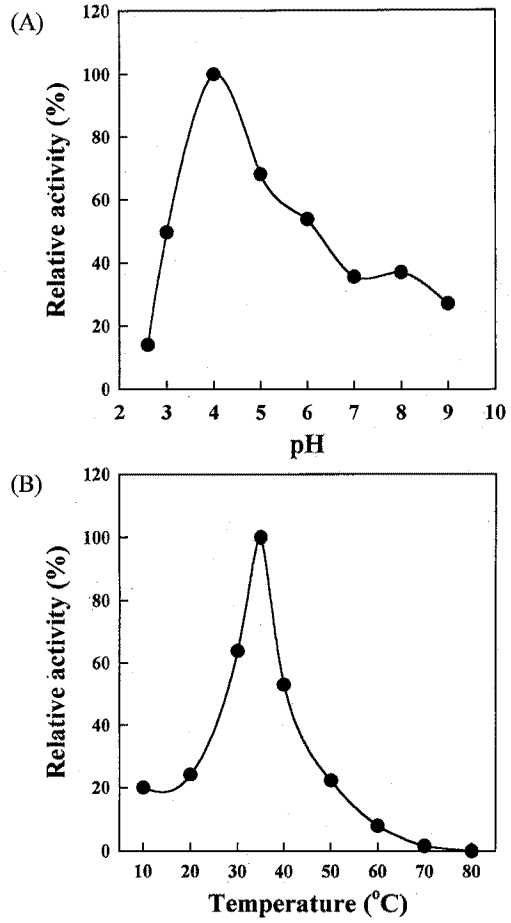


**Fig. 4.** Effect of pH and temperature on the chitinolytic activity of the purified RHBC. (A) pH, (B) temperature

**Enzymatic properties of RHBC** The optimal pH of RHBC was 4.0. About 30% of the activity remained at pH 7 to 9 (Fig. 4A). As seen in Fig. 4B, the purified chitinase has maximal activity at 35°C. This relatively lower and narrow range of optimal temperature is similar to that of wheat germ (Molano *et al.*, 1979), while most plant chitinases are stable up to 50°C (Collinge *et al.*, 1993). β-*N*-Acetylglucosaminidase or lysozyme activity was not observed even after prolonged incubations. Although the crude extract of rice hulls had 4 to 10 times higher β-1,3-glucanase activity than other parts of rice seed (Han *et al.*, 1998), the purified enzyme was devoid of this enzyme activity. The $K_m$ and $V_{max}$ of RHBC were 1.36 mg/ml and 13.5 mg/mg protein/hr, respectively (Fig. 5).

**Table 1.** Purification of RHBC from rice hulls.

| Treatment | Protein (mg) | Activity (μmol/h) | Specific activity (μmol/mg-h) | Recovery (%) | Purification (fold) |
|---|---|---|---|---|---|
| Crude | 1450 | 34.2 | 0.024 | 100 | 1 |
| Source-Q | 130 | 11.97 | 0.094 | 34 | 4 |
| Chitin affinity | 0.213 | 0.44 | 2.07 | 1.3 | 88 |

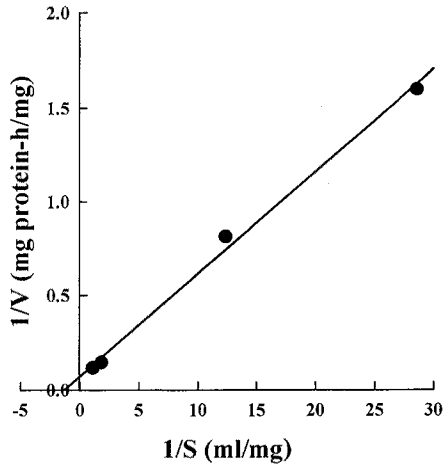


**Fig. 5.** Lineweaver-Burk plot for hydrolysis of [$^3$H]chitin by RHBC. Five hundred and eighty eight dpm correspond to 1 ug of chitin. $K_m$: 1.36 mg/ml, $V_{max}$: 13.5 mg/mg protein/h

**Table 2.** Hydrolysis of some *N*-acetyl-chitooligosaccharides by RHBC.

| Substrate | Products |
|---|---|
| $(GlcNAc)_3$ | None |
| $(GlcNAc)_5$ | $(GlcNAc)_2$, $(GlcNAc)_3$ |
| $(GlcNAc)_6$ | $(GlcNAc)_2$, $(GlcNAc)_3$ |
| $(GlcN)_6$ | None |

GlcNAc: *N*-acetylglucosamine, GlcN: glucosamine

When ρ-nitrophenyl-*N*-acetylglucosaminide and the trimer of GlcNAc were incubated with RHBC under prolonged incubations, no hydrolysis products were detected under our experimental conditions. After a 1 h incubation, the formation of dimer and trimer from the GlcNAc pentamer and hexamer was equally observed. The same accumulation of dimer and trimer from two substrates was also reported in chitinase from a green onion (Kim, *et al.*, 1992). However, this chitinase did not hydrolyze the hexamer of glucosamine (Table 2). Consequently, this lead to the conclusion that RHBC is highly specific to chitin.

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