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Oat (*Avena sativa*) Seed Extract as an Antifungal Food Preservative Through the Catalytic Activity of a Highly Abundant Class I Chitinase

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Abstract Extracts from different higher plants were screened for the ability to inhibit the growth of *Penicillium roqueforti*, a major contaminating species in industrial food processing. Oat (*Avena sativa*) seed extracts exhibited a high degree of antifungal activity and could be used directly on rye bread to prevent the formation of *P. roqueforti* colonies. Proteins in the oat seed extracts were fractionated by column chromatography and proteins in fractions containing antifungal activity were identified by liquid chromatography–tandem mass spectrometry (LC-MS/MS) and database searches. Identified antifungal candidates included thaumatin-like proteins, 1,3-beta-glucanase, permatin precursor, pathogenesis-related protein type 1, and chitinases of class I and II. Class I chitinase could be specifically removed from the extracts and was found to be indispensable for 50% of the *P. roqueforti* inhibiting activity. The purified class I chitinase has a molecular weight of approximately 34 kDa, optimal chitinase activity at pH 7, and exists as at least two basic isoforms (pI values of 7.6 and 8.0). Partial sequencing of the class I chitinase isoforms by LC-MS/MS revealed a primary structure with high similarity to class I chitinases of wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and rye (*Secale cereale*). Oat, wheat, barley, and rye seed extracts were compared with respect to the abundance of the class I chitinase and decrease in antifungal activity when class I chitinase is removed. We found that the oat seed class I chitinase is at least ten times more abundant than the wheat, barley, and rye homologs and that oat seed extracts are highly active toward *P. roqueforti* as opposed to extracts of other cereal seeds.

Keywords Oat · Chitinase · Protein purification · Antifungal · Mass spectrometry

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Introduction

The use of antifungal agents as preservatives is of high interest in industrial food processing. Fungi are able to contaminate all kinds of food including cereals, meat, milk, fruit, vegetables, nuts, fats, and products of these. Especially fungi of the genera *Penicillium*, *Aspergillus*, and *Fusarium* are involved in food spoilage in particular by secretion of highly poisonous mycotoxins [1]. A limited number of species colonize each single food type but in a highly specific manner. Strains of *Penicillium*, the target of this study, are known to be involved in spoilage of stored wheat and rye grains, rye bread, cheese, sausages, garlic, and onions as well as various types of fruit.

Antifungal agents can be smaller organic compounds but also high-molecular-weight proteins and enzymes. Proteins with antifungal properties have been isolated from bacteria, fungi, and in particular, higher plants. Most antifungal proteins are pathogenesis-related (PR) proteins [2]. PR proteins are induced in plant tissues after fungal, bacterial, and viral infections or as a result of other stress factors but are also accumulated in healthy conditions. A large number of PR proteins have been characterized and grouped into 14 classes based on characteristics like enzymatic activity, primary structure, and biological specificity [2]. Different PR proteins respond to different infection types in various tissues predominantly in supplementing or synergistic fashions [2, 3].

Cereal crops express numerous PR proteins of which members belonging to PR-1, PR-2 (β-1,3-glucanases), PR-3 (chitinases), PR-5 (thaumatin-like proteins), and PR-9 (peroxidases) have been most extensively studied [4–7]. Plant chitinases (EC 3.2.1.14) catalyze the hydrolysis of β-1,4-glycoside bonds present in chitin, a natural homopolymer of *N*-acetylglycosamine [5]. Chitin is a major component of the exoskeleton of many fungal pathogens. The chitinases are a highly complex group of enzymes expressed in different amounts and specificities depending on the type of plant tissue. Chitinases are not only responsible for defending plants against fungal attack but are also involved in processes related to growth and development. Chitinases are grouped into family 18 and 19 including class III and V and class I, II, IV, VI, and VII chitinases, respectively [8]. Class I and II chitinases are relatively abundant in cereal seeds [9]. Plant class I chitinases are composed of a chitin-binding (CB) basic, cysteine-rich N-terminal domain and a C-terminal catalytic domain while class II chitinases only have the catalytic domain [10, 11]. Class I chitinases are more active toward fungi and insoluble substrates than the class II chitinases [12, 13] and it has been suggested that the CB domain of rye seed class I chitinase (RSC-a) assists antifungal action by binding to fungal hyphae. Class I chitinases from seeds of rye (*Secale cereale*), barley (*Hordeum vulgare*), and wheat (*Triticum aestivum*) have been sequenced and biochemically characterized [7, 14].

In this study, we identify extracts of oat seeds as a potential antifungal agent for use in food preservation. Antifungal proteins in the oat seed extracts are isolated and characterized. Primary structure, abundance, and antifungal activity of class I chitinases is compared among extracts from cereal seeds.

Materials and Methods

Preparation of Extracts

Cultivars of oat, barley, rye, and wheat were obtained from Svalöf Weibull B.V.—The Netherlands (oat cv Adamo), Sejet planteforædling—Denmark (oat cv Markant), DLG—

Denmark (wheat cv Vinjett, barley cv Smilla, and barley cv unknown), FAF Odense—Denmark (wheat cv Asketis), and Kohberg A/S (rye cv unknown). Protein extracts were prepared by incubating milled seeds in water for 16 h at room temperature (400 g/L) followed by the removal of insoluble particles by filtration through a cheesecloth and centrifugation at 5,000×*g* for 15 min.

Testing Extracts on Rye Bread

Fresh rye bread was supplied from Kohberg A/S and the upper 20 mm were excised and divided into 40-mm slices. Bread slices were placed in closed plastic containers (SL-430, Færch plast—Denmark). Spores of *P. roqueforti* (approximately five spores per square centimeter) and oat seed extracts (2 μL/cm^2) were applied using a vaporizer. Containers were incubated at 25 °C in plastic bags for 7 days. Colonization by *P. roqueforti* was documented by digital photos.

Protein Purification

Antifungal activity was fractionated by Q-Separose-HP and Phe-Sepharose-HP column chromatography. Protein extracts of oat seeds (50 mL) were adjusted to the composition of buffer A (50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 10% glycerol, 0.1 mM PMSF) and chromatographed through a 20-mL Q-Sepharose-HP column. The antifungal activity which was retained in the flow-through fraction was adjusted to buffer B (buffer A+1,500 mM $(NH_4)_2SO_4$) and bound to a 20-mL Phe-Sepharose-HP column previously equilibrated in buffer B. Antifungal activity was eluted by a linear gradient from buffer B to buffer A in two peaks at 1,000 mM (fraction A) and 500 mM (fraction B) $(NH_4)_2SO_4$, respectively.

Class I chitinases were purified from the extracts by chromatography on Q-sepharose-HP as described above followed by chitin affinity chromatography. A 15-mL column packed with chitin beads (New England BioLabs) was used to bind the flow-through fraction from Q-Sepharose-HP. The column was washed extensively in buffer A and chitin-binding chitinases (class I chitinase) were eluted using 20 mM acetic acid. Neutralizing buffer (ten times concentrated buffer A) was added to the fractions immediately after elution (30% *v*/*v*).

Class I chitinase was removed from seed extracts by adding chitin beads previously washed in buffer A (5% of total volume) followed by centrifugation at 800×*g* for 5 min. Beads were washed six times in buffer A and boiled in NuPage (Invitrogen) sample buffer containing 0.1 mM 1,4-dithioreitol and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to compare the abundance of class I chitinases in cereal species.

Gel Electrophoresis

NuPage 12% gels (Invitrogen) were used for SDS-PAGE, and IEF gels (BioRad) with a separation range from pI 3 to 11 were used for isoelectric focusing. All procedures were according to the manufacturer's instructions.

Test of Antifungal Activity

The growth of *Penicillium roqueforti* IBT 5426 (Obtained from Biocentrum DTU, Denmark) was used as a measure for antifungal activity. Fungi were grown in ME+5%

NaCl (20 g/L malt extract, 1 g/L peptone, 10 mg/L $ZnSO_4 \cdot 7H_2O$, 5 mg/L $CuSO_4 \cdot 5H_2O$, 50 g/L NaCl, and 20 g/L glucose. Samples were dialyzed against ME+5% NaCl and tested in microtiter plate assays. Microtiter wells contained a total volume of 140 μL including 20 μL of the redox indicator alarmarBlue (Biosource) and 20 μL *P. roqueforti* IBT 5426 spores in ME+5% NaCl (final concentration ~4,000 spores per milliter). The remaining 100 μL contained ME+5% NaCl including the test sample. The absorbance of the redox indicator at 600 nm was measured in the wells after 72 h at 25 °C and compared for different samples. All samples were assayed in triplicate to allow calculation of standard deviations.

In Vitro Assay of Chitinase Activity and Determination of pH Optimum

Chitinolytic activity was assayed using CM-Chitin-RBV (Loewe Biochemica GmbH) as a substrate. The assay was performed as described by the manufacturer using a tricomponent profile buffer system providing constant ionic strength in the pH range from 4 to 9 [15].

Identification and Sequencing of Proteins by LC-MS/MS

Proteins were separated using SDS-PAGE. Protein bands were excised and in-gel digested with trypsin [16]. Purified class I chitinase was excised from SDS gels and in-gel digested using trypsin, chymotrypsin, proteinase K, AspN, or GluC. The resulting peptides were analyzed using a q-ToF2 (Micromass UK Ltd., Manchester, UK) coupled online to a waters capillary LC system (Waters, Milford, MA, USA). Peptide samples were loaded on a 1-cm precolumn (75 μm inner diameter) packed with ReproSil-pur AQ-C18 3 μm (Dr. Maisch, GmbH, Germany) using an autosampler. Peptides were separated using a linear gradient from solution A (2% acetonitrile, 0.1% formic acid) to 50% of solution B (80% acetonitrile, 0.1% formic acid) over the precolumn in line with a 15-cm resolving column (75 μm inner diameter) packed with ReproSil-pur AQ-C18 3 μm. The resolving column was connected to a coated fused silica emitter (20 μm inner diameter, 10 μm tip inner diameter; New Objective, Cambridge, MA, USA). The flow rate was 200 nL/min. The mass spectrometer was operated in the positive ion mode with a source temperature of 80 °C, collision energy of 25–35 eV, resolution of 7,000–8,000 Da. Eluted double and triple positively charged ions were selected for MS/MS analysis and protein identity and sequence information was obtained by de novo sequencing or by raw data processing using the ProteinLynx Global server software.

The partial sequence of oat seed chitinase is deposited in Swiss-Prot/TrEMBL under the accession number P86181.

Results and Discussion

Oat Seed Extracts as Food Preservatives

Extracts of different higher plant tissues were initially tested for their inhibitory influence on fungi in an attempt to identify potential candidates for the preservation of food. Experiments indicated that an aqueous extract of the oat cv Adamo performed particularly well compared to other extracts from higher plant tissues as well as other oat cvs as judged after the production of several independent batches (data not shown). The Adamo extract was found to inhibit fungal species belonging to *Penicillium*, *Aspergillus*, *Eurotium*, *Phoma*, *Trichoderma*, *Alternaria*, *Geotrichum*, *Cladosporium*, *Botrytis*, *Paecilomyces*, *Rhizopus*, and *Candida* but not *Fusarium* (data not shown).

Rye bread is, in particular, hampered by fungal pathogens, especially species of the genus *Penicillium*. This is costly in industrial production especially during hot and moist periods. We, therefore, tested the efficiency of oat seed extracts as an antifungal agent toward *P. roqueforti* spores applied onto fresh rye bread (Fig. 1). Different combinations of

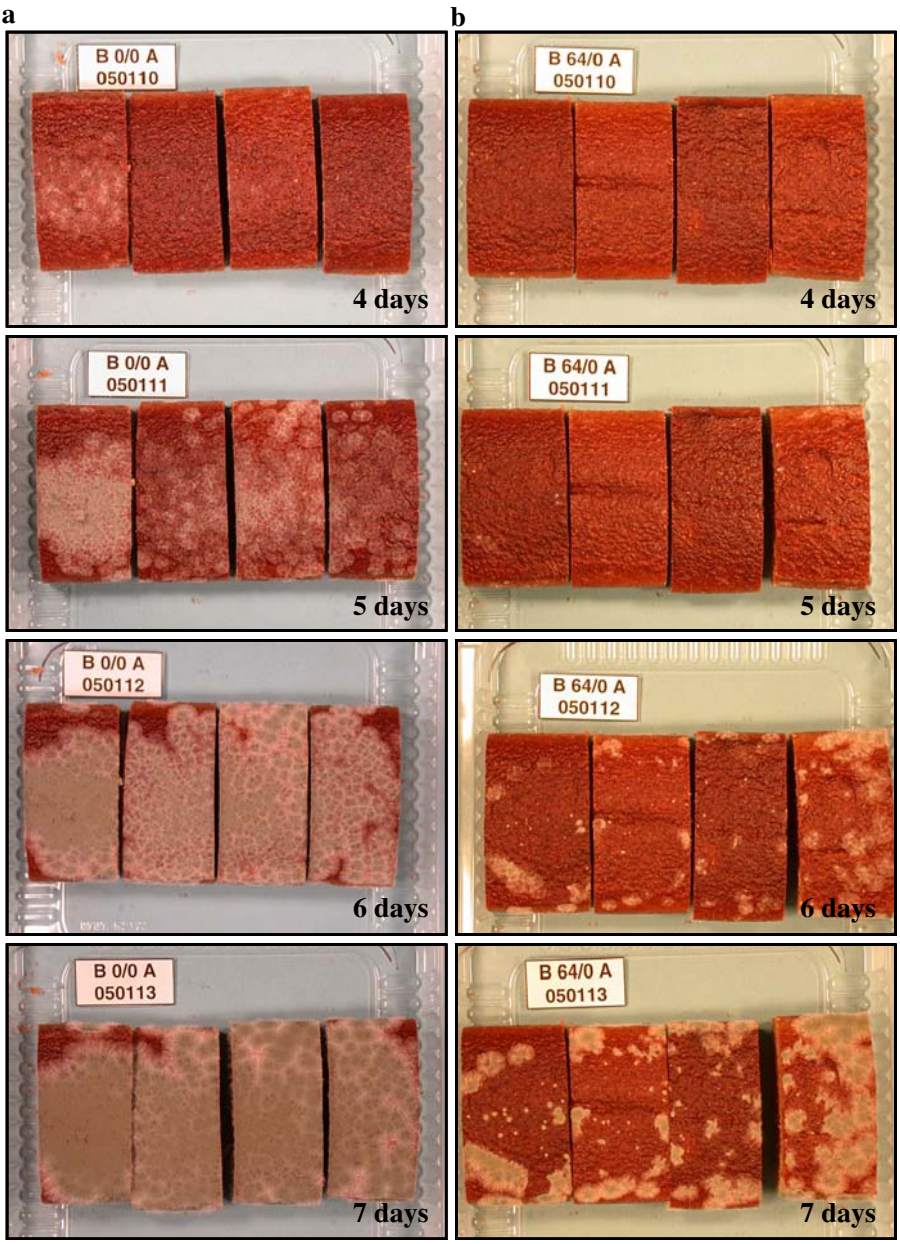

Fig. 1 Inhibition of the growth of *P. roqueforti* on contaminated rye bread by oat seed extracts. Breads were contaminated with approximately five spores per square centimeter in the absence (**a**) and presence (**b**) of oat seed extract (2 μL/cm^2) for the indicated number of days and photographed

spore and oat seed extract concentration were tested. We found that a concentration of 2 μL/cm^2 oat seed extract inhibited the growth of *P. roqueforti* on rye bread for at least 4 days at initial spore concentrations of ten spores per square centimeter (maximum concentration tested).

Identification and Isolation of Inhibitory Components

Initial experiments indicated that the majority of the antifungal activity found in the oat seed extracts required the presence of proteins. Antifungal activity disappeared when extracts were boiled or treated with proteases and was retained by filtration through 50-kDa molecular weight cut-off membranes (data not shown).

Chromatography of oat cv Adamo seed extracts through SP-Sepharose-HP and Q-Sepharose-HP columns removed 20% and 80% of the total protein, respectively, but in neither case the antifungal activity (Table 1). Contrarily, Phe-Sepharose-HP chromatography enabled binding of the total antifungal activity and elution into two major antifungal fractions. Extracts were consequently chromatographed through Q-Sepharose-HP to remove most of the nonantifungal fraction (mainly seed storage proteins) followed by separation of the antifungal activity into two fractions using Phe-Sepharose-HP (Fig. 2a). Proteins in the two antifungal fractions were identified by liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis of tryptic peptides and database searches.

The least active fraction (fraction A) contained five proteins with similarity to previously described antifungal proteins (Fig. 2b). The class II chitinase of approximately 26 kDa has been described in seeds of barley [6, 17] and rye [11] and the three-dimensional structure of the enzyme from barley has been solved [18]. Synergistic effects have been observed when the class II chitinase is combined with antifungal proteins [6]. This chitinase was found in fraction A together with a permatin precursor protein (PR-5), thaumatin-like protein (PR-5), 1,3-beta-glucanase (PR-2), and type 1 pathogenesis-related protein (PR-1). The function and mode of action of the latter antifungal protein type (PR-1) is less well-understood [2]. The oat permatin protein is an antifungal thaumatin-like protein of PR-5 with rather complex functional patterns [2, 19].

The proteins present in fraction A were approximately 50% less active than the proteins in fraction B (Fig. 1a). A thaumatin-like protein (PR-5) was eluted in fraction B along with an approximately 34-kDa class I chitinase.

The class I chitinase was the most abundant protein in the antifungal fractions A and B and it was speculated if this protein represents the majority of the antifungal activity in the seed extracts of oat cv Adamo.

Table 1 Data from purification steps.

	Amount of protein (mg)	Chitinase activity (A595/mg)	Antifungal activity—IC_{50} (mg/mL)
Oat seed extract	175.2	65.5±9.5	1.7
After Q-Sepharose-HP	28.8	446.3±77.3	0.12
HIC fraction A	3.1	651.4±31.1	0.15
HIC fraction B	5.3	476.9±39.8	0.08

The variation of antifungal activity was within 10%

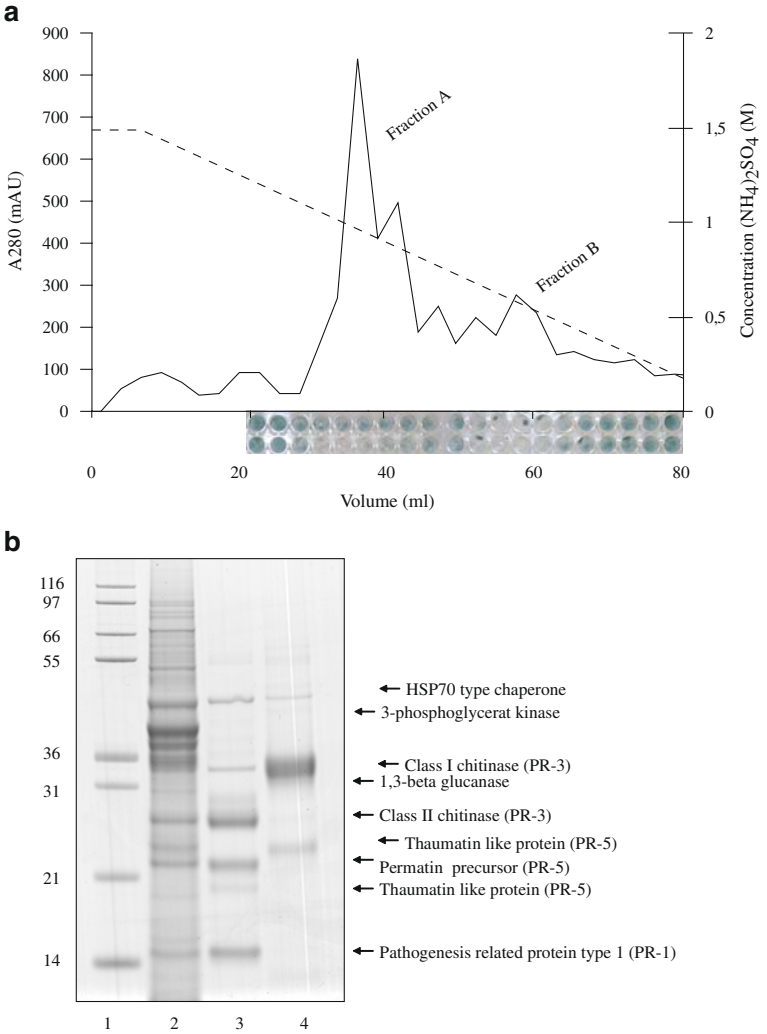

Fig. 2 Isolation of active components from oat cv Adamo seed extracts. **a** Chromatography on Phe-Sepahrose-HP using oat seed cv Adamo extract previously chromatographed on Q-Sepharose-HP. Two antifungal fractions (*A* and *B*) are eluted at 1 and 0.5 M $(NH_4)_2SO_4$, respectively. The antifungal microtiter assay is shown for fractions corresponding to elution positions in the chromatogram. *P. roqueforti* spores were inoculated in two different concentrations (4,000 spores per milliliter, *upper row* and 400 spores per milliliter, *lower row*). **b** 12% SDS-PAGE: *lane 1* mark 12 molecular weight standard (Invitrogen), *lane 2* seed extract from oat cv Adamo seed extracts, *lane 3* fraction A, *lane4* fraction B. Bands corresponding to proteins identified by LC-MS/MS and database searches are indicated by *arrows*

Class I Chitinase of Oat Seeds

The proportion of antifungal activity in two different oat seed extracts catalyzed by the class I chitinase was assessed by specific removal and by purification of the chitinase from the extracts. Seed extracts from oat cv Adamo and another oat cultivar, Markant, were used. The class I chitinase could be efficiently removed with less than 10% remaining in the extracts (data not shown) and purified using chitin beads and column chromatography

(Fig. 3a). The two oat seed extracts showed no significant difference in antifungal activity. Removal of the class I chitinase resulted in an approximately 50% reduction in the antifungal activity (Fig. 3b). Purified class I chitinase was only modestly active and the activity could only be detected by visual inspection. We, therefore, suggest that this enzyme works in synergy with or require other components in the seed protein extracts. Presence of the oat seed class I chitinase is required for approximately 50% of the antifungal activity found in oat seed protein extracts. These proportions were similar for oat cultivar Adamo as well as Markant. Rye seed class I chitinase has been demonstrated to inhibit fungal growth more effectively than rye seed class II chitinase [7]. The class II chitinase in the oat seed extracts is highly abundant (Fig. 2b) and may represent a large fraction of the residual antifungal activity when the class I chitinase is removed from the oat seed extracts.

The influence of pH on the chitinolytic activity in oat cv Adamo and Markant seed extracts and for the two purified class I chitinases were determined (Fig. 3c). Chitinolytic

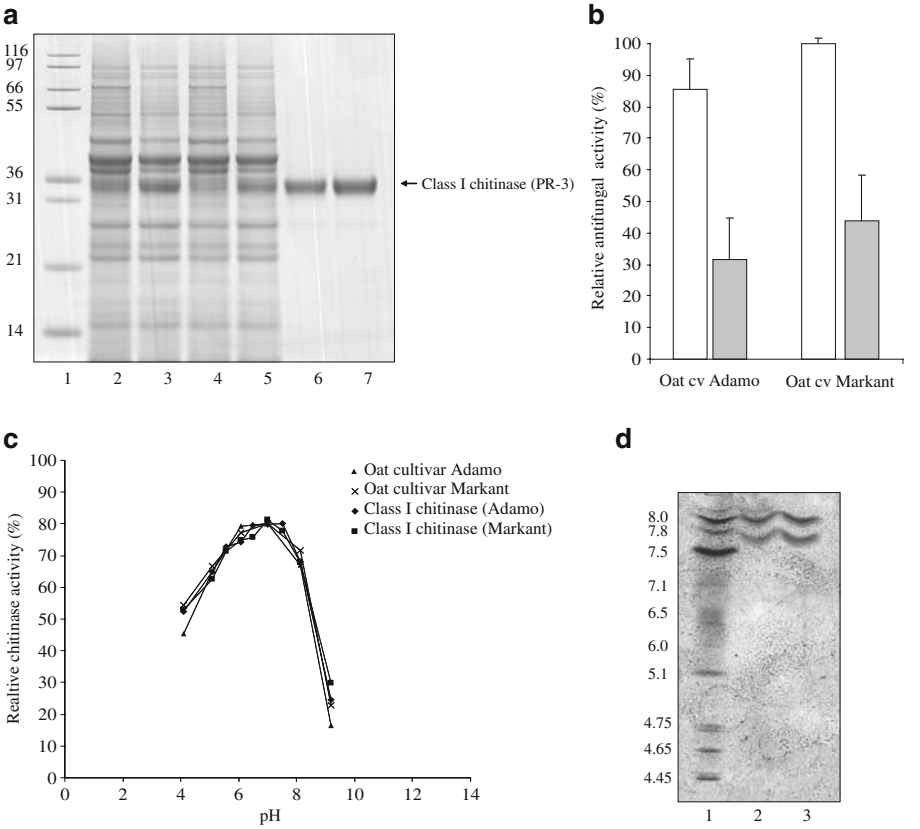

Fig. 3 Isolation and characterization of class I chitinase. **a** 12% SDS-PAGE: *lane 1* mark 12 molecular weight standard (Invitrogen), *lane 2* seed extract from oat cv Adamo, *lane 3* seed extract from oat cv Markant, *lane 4* seed extract from oat cv Adamo treated with chitin beads, *lane 5* seed extract from oat cv Markant treated with chitin beads, *lane 6* purified class I chitinase from seeds of oat cv Adamo, *lane 7* purified class I chitinase from seeds of oat cv Markant. **b** Relative antifungal activity in the samples in *lanes 2–5* of **a** (*1* lane 2, *2* lane 3, *3* lane 4, *4* lane 5). **c** Influence of pH on chitinolytic activity in seed extracts and in purified proteins. **d** IEF gel (pH 3–10) showing *lane 1* IEF marker (BioRad), *lane 2* purified class I chitinase from seeds of oat cv Adamo, *lane 3* purified class I chitinase from seeds of oat cv Markant

activity reaches its maximum at pH 7 for extracts as well as purified enzymes. The activity in all four samples drops rapidly at pH values around 8 but is only reduced by approximately 40% at pH 4. As evident from the pH influence on activity, the purified class I chitinase performs similarly to the chitinolytic activity in the extracts and the results are similar for both oat cultivars. Based on the removal of class I chitinase from extracts of Adamo and Markant cvs that reduces antifungal activity by similar factors (Fig. 3b) and the overlapping dependence of pH, we suggest that the activities are highly similar, if not identical. Isoelectric focusing revealed two basic isoforms (pI 7.6 and 8.0) present in the class I chitinase preparations from both oat cv Adamo and Markant seed extracts. There probably exists more than two class I chitinase isoforms in oat seeds. Two class I chitinase sequences are available from barley (accession numbers P11955 and Q42839), two from rye (accession numbers Q9FRV1 and Q9AXR9), and five from wheat (accession numbers Q8W427, Q9XEN6, Q6T484, Q8W428, and Q41539). In this study, we present the partial sequence of oat seed class I chitinase obtained by de novo sequencing of proteolytic peptides using mass spectrometry (P86181) (Fig. 4). The sequence presented in Fig. 4 only includes the peptides obtained from both of the oat seed chitinase isoforms (pI 7.6 and 8.0). Peptides belonging to specific isoforms were also found but their assembly and annotation to the correct isoform was difficult and uncertain. We recommend that the sequence of oat seed chitinase isoforms is obtained by cDNA cloning and DNA sequencing. However, this was not the aim of the present study. The catalytic region of oat seed class I chitinase is highly similar to that from selected sequences of rye, wheat, and barley. The two potential catalytic residues E126 and E148 (for rye class I chitinase nomenclature, see refs. [20, 21]) are also present in the oat seed class I chitinase. The size of the oat seed class I chitinase

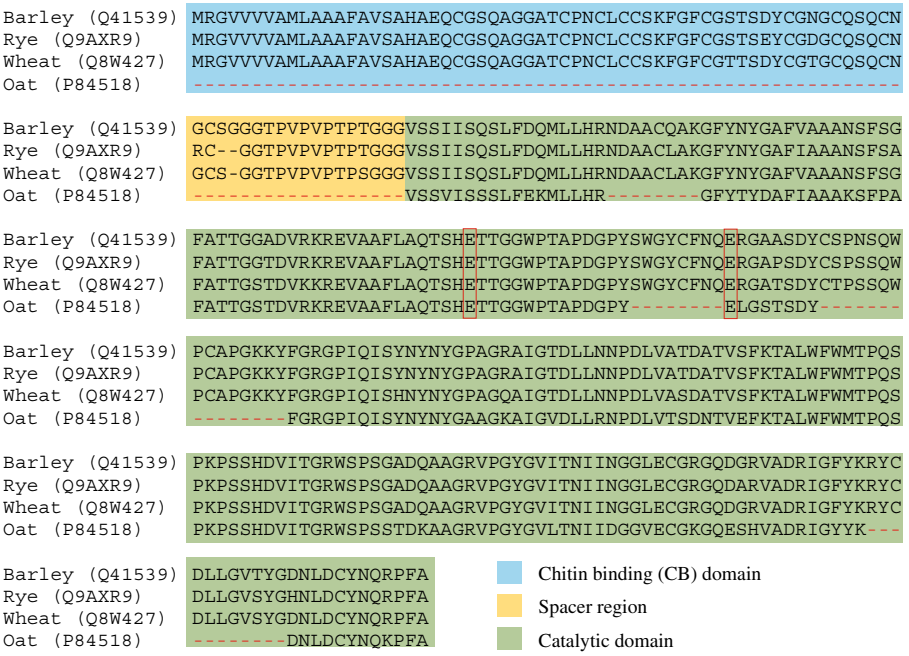

Fig. 4 Alignment of class I chitinase sequences from cereal seeds. The partial sequence of the oat chitinase was determined by LC-MS/MS. The catalytic residues E126 and E148 are indicated by a *rectangular marking*. *Dotted lines* represent regions that could not be sequenced

(approximately Mr 34 kDa) and its ability to bind chitin beads suggests that the enzyme contains a CB domain similar to that found in other cereal class I chitinases.

In conclusion, the class I chitinase from seed extracts of the oat cvs Adamo and Markant consists of at least two isoforms. Removal of the class I chitinase isoforms reduces the total antifungal activity by approximately 50%. Both isoforms are identical among the two oat cvs with respect to the sequenced peptides (Fig. 4).

Class I Chitinase in Cereal Seeds

To evaluate the distinctiveness of the antifungal properties possessed by the oat seed extracts and the identified class I chitinase, we compared the antifungal performance and abundance of class I chitinase among cereal seeds. Class I chitinases were removed from seed extracts of oat cvs Adamo and Markant as well as two wheat cvs, two barley cvs, and one rye cv. The extracts from the different species were compared by SDS-PAGE to ensure that the protein concentrations were comparable (Fig. 5a). Proteins pulled down from the extracts by chitin beads were analyzed by SDS-PAGE and identified by mass spectrometry analysis and database searches (Fig. 5b). A protein of approximately Mr 32–35 kDa could be pulled down from all analyzed extracts. This protein was identified as class I chitinase in all cases. From these experiments, we conclude that the amount of class I chitinase that can be pulled down from oat seed extracts is at least ten times higher compared to wheat, barley, and rye seed extracts. The apparent abundance of class I chitinase in seed extracts from different species seems to be in the order oat>rye>barley>wheat. However, a higher number of cvs should be included to draw a final conclusion. The activity and abundance of

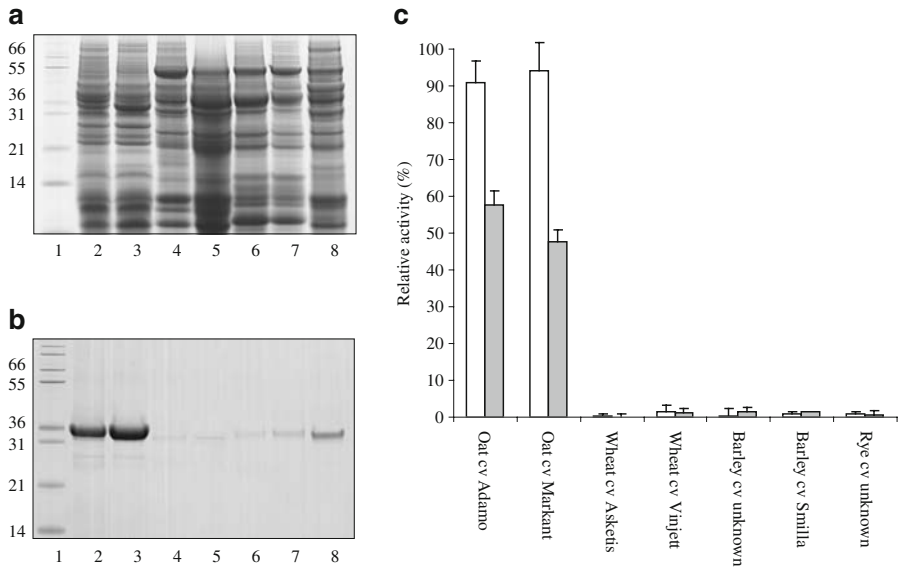

Fig. 5 Comparison of class I chitinases in seed extracts from oat, wheat, barley, and rye with respect to abundance and inhibition of *P. roqueforti*. **a** SDS-PAGE of seed extracts and **b** protein pulled down by chitin beads. Samples are: *lane 1* mark 12 molecular weight standard (Invitrogen), *lane 2* oat cv Adamo, *lane 3* oat cv Markant, *lane 4* wheat cv Asketis, *lane 5* wheat cv Vinjett, *lane 5* barley cv unknown, *lane 6* barley cv Smilla, *lane 7* rye cv unknown. **c** Inhibition of *P. roqueforti* by seed extracts (*white bars*), seed extracts treated with chitin beads (*gray bars*)

antifungal proteins is complex and merely related to the challenges encountered in natural environments [2].

Only the oat seed extracts inhibited the growth of *P. roqueforti* (Fig. 5c). These data demonstrate that oat seed extracts inhibit the growth of *P. roqueforti* efficiently as opposed to wheat, barley, and rye seed extracts. The abundance of class I chitinases and thereby increased activity in seed extracts is favorable in industrial food production and preservation. Aqueous seed extracts meet absolutely no restrictions and the use of ecological oat seeds would extend the possibilities to meet the requirements dictated by ecological consciousness.

Conclusions

The data presented in this study illustrate the application of oat seed extracts for the preservation of rye bread. A highly abundant class I chitinase is required for at least 50% of the activity that inhibits the growth of *P. roqueforti*. The inhibitory effect toward *P. roqueforti* is unique to oat seed extracts and could not be reproduced with seed extracts from cvs of wheat, barley, or rye. We recommend that oat seed extracts be used as a preservative in the bread industry and in other relevant food processing.

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