Cloning and characterization of an endo-β-1,3(4)glucanase and an aspartic protease from *Phaffia rhodozyma* CBS 6938

**Abstract** We describe the identification and expression cloning of two novel enzymes, a β-glucanase and an aspartic protease, secreted from the basidiomycetous yeast *Phaffia rhodozyma*. A cDNA library from *P. rhodozyma* CBS 6938 was constructed, and full-length cDNA encoding an endo-1,3(4)-β-glucanase (bg1) and an aspartic protease (pr1) were cloned by expression cloning in *Saccharomyces cerevisiae* W3124. The bg1 cDNA encodes a 424-residue precursor protein with a putative signal peptide. The pr1 cDNA encodes a 405-residue prepropolypeptide with an 81-residue leader peptide. The aspartic protease was purified and characterized. It has a molecular mass of 36 kDa, an isoelectric point of pH 7.5, a pH activity optimum at 4.0–6.0, and a temperature activity optimum around 40 °C. Both enzymes show only low sequence identity to other known enzymes.

**Introduction**

*Phaffia rhodozyma* is a basidiomycetous yeast found associated with slime exudates occurring at sites of wound injuries on deciduous trees. It was first isolated from the mountainous areas of Japan and Alaska by Phaff et al. in 1972. *P. rhodozyma* is red-pigmented and able to ferment sugars. (Miller et al. 1976), and is unusual as, unlike other pigmented yeasts, it synthesizes astaxanthin as the primary carotenoid pigment (An et al. 1991).

The main habitat of *P. rhodozyma* is slime fluxes of deciduous trees. We therefore expected it to secrete enzymes capable of degrading plant cell wall components, such as cellulose, pectin and hemicellulose. Such enzymes are of commercial interest in a broad variety of industries such as washing, baking, brewing, etc.

The aim of the present study was to screen for extracellular enzymes from *P. rhodozyma*, and subsequently to isolate the genes encoding such enzyme activities by expression cloning in *Saccharomyces cerevisiae* W3124. Expression cloning is suitable for the isolation of all types of enzyme activities for which a plate assay can be developed (Dalbøge and Heldt-Hansen 1994). Following this approach we cloned an endo-1,3(4)-β-glucanase and an aspartic protease from *P. rhodozyma*. These enzymes are among the first to be isolated from bacidiomycetes, as most effort has previously been put into isolating extracellular enzymes from filamentous fungi belonging to the ascomycetes. In the present paper we describe the cloning and characterization of these two enzymes.

**Materials and methods**

**Media**

BPX medium, containing (per liter) 500 g potato flour, and 250 g barley flour was degraded with 0.5 g α-amylase (BAN, Novo Nordisk A/S) by heating from 60 °C to 85 °C for 30 min. Subsequently, 50 g Sodium-caseinate was added at 60 °C, and dissolved with the potato flour. Thereafter, 100 g soy grits, 45 g Na₂HPO₄ · 12H₂O and antifoam were added and heated to boiling. The pH was adjusted to 7.5, and distilled water was added until a final volume of 1 l was reached. YP medium contains (per liter) 10 g yeast extract (Difco), 20 g Bactopeptone (Difco), and 20 g glucose or galactose. In addition, one of the following components was
added to each of the media: 7% Pharmamedia (Traders Protein, USA), 2% cellulose (Sigma), 3% Solcaflo (Novo Nordisk A/S, Denmark), 3% carboxymethylcellulose (Sigma), 3% Stärke löslisch (Merck), 2% modified hairy regions from apples (Kofod et al. 1994), 2% highly esterated pectin (DE 75%); Herbstreith and Fox KG, Neuenbürg, Germany), 2% olive oil (Sigma) or 2% jojoba oil (Sigma).

Plate screening assays

Assays for endohydrolases were carried out on plates containing 0.1% AZCL-substrate (MegaZyme, Australia). Activity created blue halos formed by the release of soluble dyed oligomers from the insoluble substrate. The following AZCL-substrates were used: AZCL-amylose, AZCL-HE(hydroxyethyl)-cellulose, AZCL-xylloglucan, AZCL-xylan, AZCL-galactan, AZCL-debranched arabinan, AZCL-curdlan, AZCL-β-glucan, and AZCL-pustulan. Screens for protease activity were performed on plates containing 0.5% casein in 0.1 M citrate/phosphate buffer, pH 5.5, and proteolytic activity was identified by the formation of white halos caused by the cleavage of casein. Assays for pectinase activity were performed on plates containing 1% highly esterified apple pectin (DE 75%) in 0.1% citrate/phosphate buffer, pH 6.0. Activity was revealed after incubation for 24 h by precipitation with 1% mixed alkylltrimethylammonium bromide. Pectin methyl esterase activity gives a turbid pectate precipitate, while pectin lyase activity gives a turbid precipitate. Pectin methyl esterase activity is also present. In this case polygalacturonase activity will be seen as clearing zones. Lipase activity was assayed on plates containing 0.1 M sodium citrate, 0.1 M H3PO4, pH 5.5, was used and the gel was incubated at 30 °C for 0, 1, 2, 4 and 24 h, followed by heat inactivation at 95 °C for 15 min. Samples containing 25 μl each supernatant from the samples were subjected to HPLC analysis. HPSEC with refractive-index detection was performed as described previously (Christgau et al. 1995).

Expression cloning

A P. rhodozyma strain, CBS 6938 was grown in 250 ml BPX growth medium. The cells were harvested after 2 days of growth at 20 °C, frozen in liquid N2 and stored at −80 °C. Construction of a cDNA library in pYES 2.0 (Invitrogen, USA) was carried out as previously described (Draborg et al. 1995). The library consisting of 3.2 x 10^7 clones was stored as individual pools (7000–10 000 colonies each pool) in 20% glycerol (pH 8.0) at −80 °C. Plasmid DNA from a cDNA library pool was transformed into Saccharomyces cerevisiae, W3124 (Hazel et al. 1992) by electroporation (Becker and Guarante 1991) and plated on SC plates (Sherman 1991) containing 2% glucose. After incubation at 30 °C for 3 days, the colonies were screened for arabinanase, β-glucanase and protease activity by replica plating onto assay plates. Clones expressing arabinanase and β-glucanase were selected by replication onto SC plates containing 2% galactose and 0.1% AZCL-arabinanase or 0.1% AZCL-β-glucan (MegaZyme, Australia) respectively. Selection for clones expressing protease activity was carried out by replicating colonies onto SC plates containing 2% galactose. After 4 days of incubation at 30 °C, the colonies were assayed for protease activity by incubation with 1% agarose overlay containing 0.5% casein in 0.1 M sodium citrate, 0.1 M NaHPO4, pH 5.5. Following overnight incubation at 30 °C, positive clones were identified and isolated from the master plates. Positive clones were repurified twice, and transformed into E. coli DH5α (Gibco, BRL). Subsequently, the insert-containing pYES 2.0 plasmid was purified by the Qiagen method (Qiagen, Germany).

Nucleotide sequence analysis

The nucleotide sequences of the cDNA clones were determined from both strands by the dye-deoxy-chain-termination method (Sanger et al. 1977) using fluorescently labeled terminators. Qiagen-purified plasmid DNA (Qiagen, Germany) was sequenced, using the Sequenase kit (United States Biochemical, USA) with either T7 E. coli polymerase primers (Invitrogen) or synthetic oligonucleotide primers and an ABI PRISM 377 DNA sequencer according to the manufacturer's instructions. Sequences were analyzed using programs available with the Genetics Computer Group (GCG) software package (Wisconsin Package Version 8.0, University of Wisconsin Biotechnology Center, Madison, Wis., USA) (Devereux et al. 1984).

Electrophoresis

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Mini-Leaf 4 electrophoresis unit (Kem-En-Tec, Denmark) in a modified version of the Laemmli procedure (Laemmli 1970; Christgau et al. 1991). Molecular mass markers (Merck) were run on each gel and the positions of the markers were calculated using Pharmacia’s imaging software. Electrophoresis was carried out at 20 °C, with 20 ml samples being loaded onto each gel. Proteins were transferred to Polyvinylidene fluoride (PVDF) membranes using the Trans-blot (Bio-Rad) dry blotter and the membranes were stained with Coomassie brilliant blue R-250 (Serva, Germany) staining in 40% ethanol and 5% acetic acid according to standard protocols (Matsudaira 1989). For detection of protease activity in isoelectric focusing gels, an overlay of 0.5% azoarcin blue was used in the focusing gel, 0.1 M sodium citrate, and 0.1% H2PO4, pH 5.5, was cast over the gel, and incubated at 30 °C until white zones occurred. For detection of β-glucanase activity an overlay of 0.1% AZCL-β-glucan (MegaZyme, Australia) in citrate/phosphate, pH 5.5, was used and the gel was incubated at 30 °C until blue zones appeared.

High-pressure size-exclusion chromatography (HPSEC) analysis of β-glucan degradation products

Aliquots of 0.1 ml 0.5% β-glucan substrate solution in 0.1 M acetate buffer, pH 5.0, were incubated with 20 μl P. rhodozyma culture supernatant having β-glucanase activity. The incubations were done at 30 °C for 0, 1, 2, 4 and 24 h, followed by heat inactivation at 95 °C for 15 min. Samples containing 25 μl each supernatant from the samples were subjected to HPLC analysis. HPSEC with refractive-index detection was performed as described previously (Christgau et al. 1995).

Purification of P1 from P. rhodozyma

The aspartic protease was purified from the culture supernatant of P. rhodozyma grown in YPD medium. After 4 days of incubation, 1.5 l culture supernatant was collected and concentrated to 400 ml in a 400-ml 25-kDa Amicon ultrafiltration device into 25 mM sodium acetate, pH 5.0. The sample was loaded at 4 ml/min on an HR 16/10 column containing bacta-agarose activity was specifically binds proteases. Following washing, bound proteins were eluted with 25 mM sodium acetate, pH 5.0, and 2 M NaCl in 25% isopropanol. Fractions containing protease activity were pooled, changed into 25 mM citrate, pH 4.0, by ultrafiltration and loaded onto an HR 16/10 Fast-Flow S-Sepharose cation-exchange column (Pharmacia, Sweden) at 2.0 ml/min. Bound proteins were eluted with a linearly increasing NaCl gradient from 0 to 0.5 M NaCl over 45 min. The aspartic protease eluted at approximately 0.1 M NaCl, and was