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Purification, kinetic characterization and properties of a novel thermo-tolerant extracellular protease from *Kluyveromyces marxianus* IFO 0288 with potential biotechnological interest

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HIGHLIGHTS

- A novel extracellular protease was purified and characterized kinetically.
- Optimum k_{cat}/K_m-value of novel protease was found at pH 7.75 and 35.5 °C.
- This enzyme maintained full activity at 25 °C after 30 min incubation at 10 or 60 °C.
- The K_m-value of novel enzyme was decreased at increased [Ca^{2+}] and/or [NaCl].
- All proton inventories suggest that catalysis proceeds via a charge-relay system.

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ABSTRACT

A novel extracellular hydrolase of ~45 kDa molecular mass was purified from *Kluyveromyces marxianus* IFO 0288 cultures and characterized as serine protease. The K_m-value of protease (designated protease-*KM*-IFO-0288-A), which was found active in media containing elevated [NaCl] but lacking $EDTAK_2$, decreased with increasing [Ca^{2+}]. The protease maintained considerable activity at the range of 10–60 °C and pH 6.00–10.25, with optimum k_{cat}/K_m-value at 35.5 °C and pH 7.75. It was strongly affected by specific irreversible inhibitors of serine proteases while was unaffected by inhibitors of cysteine proteases. Significant rate constants, activation energies, and proton inventories were estimated from the profiles of Michaelis–Menten parameters, versus pH, temperature and deuterium atom fraction, in the hydrolysis of Suc-AAPF-pNA showing that protease-*KM*-IFO-0288-A performs catalysis via a charge-relay system. The properties of protease-*KM*-IFO-0288-A suggest that *K. marxianus* represents a valuable source of extracellular protease of biotechnological interest which, given its GRAS status, could find several important applications.

1. Introduction

Strains belonging to the yeast species *Kluyveromyces marxianus* are commonly found in a large variety of dairy products e.g. kefir grains, soft cheeses, aged cheeses (Corbo et al., 2001; Romano et al., 2001), while they have been isolated from a variety of other habitats (Fonseca et al., 2008). It is likely that *K. marxianus* contributes to the development of functional and sensorial properties of a variety of final industrial products (Fonseca et al., 2008). Several biotechnologically important applications have been investigated with this yeast, e.g. production of enzymes, single-cell protein, ethanol, reduction of lactose content in food products, production of bio-ingredients from cheese whey, heterologous protein production, and many others (Kourkoutas et al., 2002; Fonseca et al., 2008; Padilla et al., 2012; Arrizon et al., 2012). Due to its GRAS status (generally regarded as safe), *K. marxianus* is exploited industrially in the production of aroma compounds and a large number of

Abbreviations: DMF, dimethyl formamide; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; E-64, 1-[[N-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]amino]-4-guanidinobutane; $EDTAK_2$, ethylene-diaminetetraacetate bi-potassium salt; *K. marxianus*, *Kluyveromyces marxianus* (Hansen) van der Walt IFO 0288; PMSF, phenyl-methyl-sulfonyl-fluoride; Suc, Succinyl; Suc-AAA-pNA, *N*-succinyl-L-Ala-L-Ala-L-Ala-*p*-nitroanilide; Suc-AAPF-pNA, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide; S.I.E., solvent isotope effect(s).

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enzymes with food applications, such as pectinases, polygalactoronases, inulinase and β-galactosidase which are produced as extracellular products (Fonseca et al., 2008).

Although the bibliography on enzymes produced by strains of *K. marxianus* is currently expanding, it appears to be rather limiting when it concerns the production of proteases. Zavala and coworkers (2004) reported on the purification and characterization of a serine carboxypeptidase and a lysine aminopeptidase from *K. marxianus* strains, both having a strong application potential. However, despite its potential industrial relevance, there is almost no information on the biochemistry and genetics of the proteolytic system of *K. marxianus*.

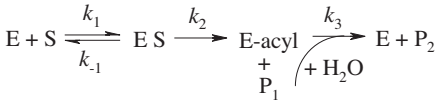
Proteases catalyze the cleavage of peptide bonds in proteins. They constitute a large family of enzymes (EC 3.4), which is divided in the endopeptidases (proteinases) (EC 3.4.21-99) and exopeptidases (EC 3.4.11-19) according to the point at which they cleave peptide chains (Beynom and Bond, 1989). Proteases share important roles in biological processes and have wide commercial applications, accounting for approximately 60% of the total worldwide market of enzymes (Barrett et al., 1982). In general, extracellular proteases are important for the hydrolysis of external proteins and enable the cell to internalize and assimilate the hydrolyzed products, while intracellular proteases play a critical role in several cellular and metabolic processes (Rao et al., 1998). Although proteases hydrolyze amide or ester bonds according to the well known three-step mechanism as shown in Scheme 1, the detailed kinetics and mechanisms of these enzymes often need to be further investigated with view to selected applications since controlled proteolysis is essential for stable qualities in industrial and biotechnological products (Nielsen, 2002).

The present work reports on the purification and detailed kinetic characterization of a novel extracellular enzyme isolated from the yeast *K. marxianus* IFO 0288, designated as protease-*KM*-IFO-0288-A. The enzyme is a thermo-tolerant serine protease, whose activity is enhanced in media containing [Ca^{2+}], and/or [NaCl] up to3.0 M. Its properties therefore may suggest an exceptional character for *protease-KM*-IFO-0288-A and a useful enzyme in biotechnological applications where high salt concentrations are be used, and/or in the development of environment friendly technologies in the food industry as an alternative of proteases from animal-sources, given the GRAS status (generally regarded as safe) of the producer microorganism.

2. Methods

2.1. Materials

The substrates Suc-AAPF-pNA and Suc-AAA-pNA, the inhibitors 3,4-dichloro-isocoumarine, PMSF and E-64, as well as other chemicals and organic solvents were of analytical grade and purchased from Sigma (U.K). HiTrap Benzamidine FF (high sub) column and Sephadex G-25 chromatography medium was purchased from GE Healthcare Bio-Sciences AB (U.S.A). All reagents for SDS/polyacrylamide gel electrophoresis, as well as the molecular mass standards were purchased from Bio-Rad (CA. U.S.A.), and from Fermentas-L.S (U.S.A.), respectively. Culture media were purchased from LAB-M (U.K.).



Scheme 1. Minimal kinetic mechanism for catalysis by proteases.

2.2. Strain, culture, and treatment of cell-free supernatants

The strain of *K. marxianus* (Hansen) van der Walt IFO 0288 was used throughout this work. Stocks were maintained in 50% (w/v) aqueous glycerol solution at −80 °C. The microorganism was grown on yeast medium (YM) agar slopes at 30 °C. Slopes containing active yeast cells were used to inoculate conical flasks containing 50 ml of in a culture medium consisting of (g/l): glucose 10.0, YNB (yeast nitrogen base without amino acids and ammonium sulfate) 5.0, and bovine serum albumin 0.4. Sterilization was done at 121.1 °C for 15 min. The flasks were incubated in an orbital shaker incubator at 150 rpm and 30 °C for 48 h. Cell growth was determined by measurement of optical density at 600 nm (OD_{600} nm). The culture broth was centrifuged at 6000 rpm at 4 °C for 20 min, and the supernatants were used for protease isolation and purification.

2.3. Isolation and purification of an extracellular protease

2.3.1. Gel-filtration chromatography

All purification procedures were carried out at 4 °C. The resulting from centrifugation cell-free supernatant was adjusted to 80% of saturation by adding ammonium sulfate and the resulted suspension was centrifuged at 9000 rpm and 4 °C for 20 min. The supernatant was discarded and the pellet was dissolved in 0.05 M Tris–HCl buffer (pH 7.40). The protein fraction obtained was loaded onto a glass column of 2.5 cm diameter and 50 cm height (Pharmacia, U.S.A.), filled with Sephadex G-25 medium and equilibrated with 1.5 l of Tris–HCl buffer 0.05 M, pH 7.40. Fractions of 5 ml were collected at a rate of 35 ml/h and were assayed for protein content and protease activity (D'Avila-Levy et al., 2003; Theodorou et al., 2001). Protease active fractions were collected and combined (55 ml), and were then subjected to affinity interaction chromatography.

2.3.2. Affinity chromatography

The pooled active fractions were further purified using an ÄKTA FPLC system (GE Healthcare Bio-Sciences AB, Sweden) equipped with a HiTrap Benzamidine FF (high sub) column. The column was washed with five volumes of distilled water to remove the storage buffer and then was equilibrated with five volumes of binding buffer 0.05 M Tris–HCl, 0.5 M NaCl, pH 7.40. The sample was loaded on the equilibrated affinity column with a flow rate of 3 ml/min; after that, the column was washed with five volumes of binding buffer to remove all unbound materials. Before any further elution, a high salt wash step with NaCl 0.75 M was performed to wash out proteins bound due to ionic forces. Active proteases were eluted with a pH gradient (CIEX buffer: 0.03 M HCOONa, 0.06 M CH_3COONa and 0.03 M Na_2HPO_4, in the range pH 7.40–2.80), at a flow rate of 3 ml/min and collected in fractions of 1.5 ml. Quantities of 60–200 μl of 1 M Tris–HCl buffer, pH 9.00 were added per ml of each collected fraction in order to prevent denaturation effects of the collected protease at low pH. All collected fractions were analyzed for protein content and proteolytic activity, while fractions with higher protease activity were pooled and used for further treatment.

2.4. Polyacrylamide gel electrophoresis

SDS–PAGE was performed as described by Laemmli (Laemmli, 1970) using a Bio-Rad (U.S.A.) Mini-Protean II gel apparatus. Aliquots of the enzyme preparations, as well as molecular mass standards were combined with sample buffer containing 100 mM Tris–HCl, 10% (v/v) glycerol, 20% (w/v) SDS (sodium dodecyl sulfate), 5% (w/v) 2-mercaptoethanol and 5% (w/v) bromophenol blue at pH 6.80. The samples were heated at 98 °C for 5 min, incubated