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Heterologous production and characterization of a chlorogenic acid esterase from *Ustilago maydis* with a potential use in baking

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ABSTRACT

Ustilago maydis, an edible mushroom growing on maize (*Zea mays*), is consumed as the food delicacy *huitlacoche* in Mexico. A chlorogenic acid esterase from this basidiomycete was expressed in good yields cultivating the heterologous host *Pichia pastoris* on the 5 L bioreactor scale (reUmChlE; 45.9 U L^{-1}). In contrast to previously described chlorogenic acid esterases, the reUmChlE was also active towards feruloylated saccharides. The enzyme preferred substrates with the ferulic acid esterified to the O-5 position of arabinose residues, typical of graminaceous monocots, over the O-2 position of arabinose or the O-6 position of galactose residues. Determination of k_{cat}/K_m showed that the reUmChlE hydrolyzed chlorogenic acid 18-fold more efficiently than methyl ferulate, *p*-coumarate or caffeate. Phenolic acids were released by reUmChlE from natural substrates, such as destarched wheat bran, sugar beet pectin and coffee pulp. Treatment of wheat dough using reUmChlE resulted in a noticeable softening indicating a potential application of the enzyme in bakery and confectionery.

1. Introduction

Chemical additives, such as emulsifiers or oxidants, are used in bread making for decades to adjust the variations of flour quality and processing parameters (Caballero, Gómez, & Rosell, 2007). In recent years, the baking industry started to replace them by enzymes. (Haros, Rosell, & Benedito, 2002). Nowadays, due to increasing mechanization in the baking process and the increasing demand for natural products, enzymes have gained a key role. The treatment of flours with enzymes, such as α-amylases, transglutaminases, glucose oxidases, xylanases and peptidases results in a changed dough structure, improved dough handling, specific volume, water retention, crumb structure, fresh bread quality and shelf life (Haros et al., 2002; Hilhorst et al., 1999; Romanowska, Polak, & Bielecki, 2006).

Especially cereal flour pentosans are known to affect distinctly the quality of dough and bakery products (Rouau, 1993). These non-starch polysaccharides mainly consist of arabinoxylans (AX) with a linear backbone of 1,4-linked β-D-xylopyranosyl units, of which up to 50% are substituted at either O-2, O-3 or both positions by α-L-arabinofuranose residues (Faulds et al., 2003). Some of the arabinose residues on the AX backbone are further O-5 esterified with ferulic acid. Wheat AX are classified into two types: One-fourth to one-third are easily soluble in water (WEAX), while the remainder are water-insoluble AX (WUAX). WUAX were reported to decrease the bread volume (Krishnarau & Hoseney, 1994), whereas the presence of WEAX affected the dough positively by increasing the viscosity due to their high water binding capacity (Rouau, 1993). The reasons for the negative effect of WUAX on the baking products are not completely understood. One explanation is related to their detrimental effect on the gluten network formation caused by the competition for water and the destabilization of the gas cells during dough development (Goesaert et al., 2005). To modify the AX structure, xylanases (EC 3.2.1.8) were introduced in the 1970 s in the baking sector (Miguel, Martins-Meyer, Figueiredo, Lobo, & Dellamora-Ortiz, 2013). They hydrolyze the AX backbone, causing a partial degradation of the AX network. Depending on the type of baked product, on dough processing and on dough properties, xylanases specific for WUAX or rather for WEAX are required. In bread-making, most commonly xylanases are used with preferential activity for WUAX. The solubilization of WUAX causes a redistribution of water to the gluten and starch phases and an increase in dough viscosity due to the formation of

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soluble AX (SAX). Consequently, these changes improve dough handling properties and stability, oven spring and specific loaf volume (Courtin & Delcour, 2001; Hilhorst et al., 1999; Rouau, 1993). In contrast, xylanases used in dry cereal products, such as biscuits, crackers and wafers, should be specific for WEAX and SAX to produce extensible doughs with a decreased resistance and viscosity (Aehle, 2007).

Another possibility to change the AX structure and subsequently their water binding capacity is the hydrolysis of side chains of the AX. Over the last years, a number of enzymes have been identified in bacteria and fungi hydrolyzing the ester linkage between ferulic acid and complex plant cell wall polysaccharides, such as arabinose substituents on AX (Williamson, Faulds, & Kroon, 1998). One group of these enzymes are feruloyl esterases (FAE; EC 3.1.1.73), a subclass of the carboxylic ester hydrolases (EC 3.1.1.-).

Recently, a chlorogenic acid esterase (EC 3.1.1.42) was isolated from the basidiomycete *Ustilago maydis* (Nieter et al., 2015). The substrate screening showed that the UmChlE was not solely active on chlorogenic acid as previously published chlorogenic acid esterases (Asther et al., 2005; Benoit et al., 2007; Schöbel & Pollmann, 1980a,b), but hydrolyzed also typical FAE substrates, such as methyl ferulate, *p*-coumarate and caffeate, as well as destarched wheat bran (DSWB) and feruloylated saccharides. Furthermore, a synergistic interaction of UmChlE with carbohydrases, such as xylanases and pectinases, was observed for the enzymatic hydrolysis of DSWB and sugar beet pectin (SBP). The full length sequence of *CHLE* was amplified and the enzyme was recombinantly produced in *P. pastoris*.

In this study, the production of the reUmChlE was scaled up in a 5 L bioreactor. Subsequently, the enzyme was purified and characterized to compare its properties with the native enzyme. As the native enzyme released ferulic acid from AX in DSWB, the effect of this enzyme on dough rheology was investigated using the Kieffer dough and gluten extensibility rig. The reUmChlE was also combined with a xylanase to determine the effect on the specific loaf volume.

2. Materials and methods

2.1. Chemicals and substrates

All chemicals were purchased in the required purity grade from Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland) or Sigma-Aldrich (Taufkirchen, Germany), unless otherwise stated. Methyl and ethyl ferulate (99%; MFA and EFA, respectively) were obtained from Alfa Aesar (Karlsruhe, Germany). Methyl caffeate (MCA), *p*-coumarate (M*p*CA), and sinapate (MSA) were synthesized according to Borneman, Hartley, Morrison, Akin, and Ljungdahl (1990). Feruloylated saccharides (5-O-*trans*-feruloyl-L-arabinofuranose (F-A); 2-O-*trans*-feruloyl-α-L-arabinofuranosyl-(1 → 5)-L-arabinofuranose (Ara_2F); 6-O-*trans*-feruloyl-β-D-galactopyranosyl-(1 → 4)-D-galactopyranose (Gal_2F); β-D-xylopyranosyl-(1 → 2)-5-O-*trans*-feruloyl-L-arabinofuranose (F-AX); α-L-galactopyranosyl-(1 → 2)-β-D-xylopyranosyl-(1 → 2)-5-O-*trans*-feruloyl-L-arabinofuranose (F-AXG); D-xylopyranosyl-(1 → 4)-β-D-xylopyranosyl-(1 → 3)-5-O-*trans*-feruloyl-L-arabinofuranose (F-AXX)) were prepared from destarched corn bran (Allerdings, Ralph, Steinhart, & Bunzel, 2006; Schendel, Becker, Tyl, & Bunzel, 2015). Sugar beet pectin was provided from Herbstreith & Fox (Neuenbürg, Germany), coffee "Guatemala Grande" was from Tchibo (Hamburg, Germany), and wheat bran was from Alnatura (Lorsch, Germany). PCR primers were supplied by Eurofins MWG Operon (Ebersberg, Germany).

2.2. Production of recombinant UmChlE in P. pastoris

P. pastoris GS115 was used for heterologous expression of the *CHLE* gene that encodes for the chlorogenic acid esterase from *U. maydis* (GenBank accession no. HG970190). Expression constructs consisting of the *CHLE* sequence without its native signal sequence, but a C-terminal *HIS*-tag sequence, were cloned into the pPIC9 vector (Invitrogen, Karlsruhe, Germany), as described elsewhere (Nieter et al., 2015). After transformation and selection on histidine-deficient MD plates (13.4 g L^{-1} YNB without amino acids, 400 μg L^{-1} biotin, 20 g L^{-1} glucose, 20 g L^{-1} agar, 50 mg L^{-1} each of L-glutamic acid, L-lysine, L-leucine, and L-isoleucin), 48 *P. pastoris* transformants were screened for chlorogenic acid esterase activity in 96-well-plates. For the microscale cultivation, the transformants were cultured at first in 600 μL YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose) for 36 h at 28 °C and 320 rpm. Subsequently, the cells were harvested by centrifugation (10 min, 4000*g*), washed twice with ddH_2O to remove the residual glucose from the YPD medium, and finally they were resuspended in 600 μL buffered complex BMMY medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 13.4 g L^{-1} YNB without amino acids, 400 μg L^{-1} biotin, 100 mM potassium phosphate pH 6.0, 0.5% methanol, 50 mg L^{-1} each of L-glutamic acid, L-lysine, L-leucine, and L-isoleucin) to start the expression. Culture supernatants were analyzed for enzyme activity after 24, 48, 72, 96 and 120 h. To maintain stable expression conditions, 0.5% (v/v) methanol was added every 24 h. For the optimization of the expression conditions, buffered minimal medium (BMM; 13.4 g L^{-1} YNB without amino acids, 400 μg L^{-1} biotin, 100 mM potassium phosphate pH 6.0, 0.5% methanol, 50 mg L^{-1} each of L-glutamic acid, L-lysine, L-leucine, and L-isoleucin) was also used.

For up-scaling, *P. pastoris* precultures were cultivated in 300/500 mL shake flasks containing 50/100 mL YPD medium. Precultures were grown at 28 °C on an orbital shaker (Infors AG, Bottmingen, Switzerland) at 180 rpm for 48 h and were used for inoculation of the baffled flask and bioreactor cultures to an OD_{600} of 1.

Shake flask cultivation was performed in 500 mL baffled flasks containing 100 mL BMMY medium at 180 rpm and 18 °C for 72 h. These conditions were selected as a result of optimization of the expression conditions, as described below. Daily, culture supernatants were screened for enzyme activity and 2% methanol was added to maintain induction.

For further up-scaling of the reUmChlE production in *P. pastoris*, a batch fermentation was carried out at 5 L scale in a stainless steel stirred tank reactor (KG5000, Medorex AG, Nörten-Hardenberg, Germany) with marine-type stirrer. Fermentation was performed in complex BMMY medium pH 6.0 containing 2% methanol, while the pH was not adjusted during fermentation. In the beginning, 0.1 mL L^{-1} antifoam agent (Tego KS800, Evonik Industries AG, Essen, Germany) was added to the medium. Following 16 h at 24 °C the temperature was kept at 18 °C until the end of the fermentation process (another 56 h). To maintain induction of gene expression 2% methanol was added every 24 h. The oxygen was supplied by using a constant flow of compressed air (5 L air/min), and dissolved oxygen saturation was monitored over the fermentation process using an oxygen electrode (Oxy Probe, Broadley James Corporation, Irvine, USA). By using a stirrer speed of 500 rpm the dissolved oxygen saturation was maintained throughout the process above 70% of the initial value.

2.3. Purification of recombinant UmChlE

The *P. pastoris* culture supernatant was separated from the cells by centrifugation (15,000*g*; 4 °C) and subsequently concentrated