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Activity of carbohydrate oxidases as influenced by wheat flour dough components

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ABSTRACT

The carbohydrate oxidase (COXMn) from *Microdochium nivale* may well have desired functionalities as a dough and bread improver, similarly to *Aspergillus niger* glucose oxidase (GOX). COXMn catalyses the oxidation of various monosaccharides as well as maltooligosaccharides for which the best activity is obtained towards the maltooligosaccharides of polymerisation degrees 3 and 4. For the same activity towards glucose under air saturation, we show that COXMn exhibits a similar efficiency towards maltose as GOX towards glucose whatever the oxygen supply. Assays with COXMn show that no competition exists between carbohydrates naturally present in the wheat flour. We show that reaction products (D-glucono-δ-lactone and hydrogen peroxide) and the wheat flour dough component, ferulic acid, have no noticeable specific effect on the COXMn activity. The demonstrated differences in kinetics between COXMn and GOX allow predicting of differences in the functional behaviours of those enzymes during wheat flour dough formation.

1. Introduction

In bread making, molecular oxygen from the air is well-recognised for its oxidative role, mainly on the free polyunsaturated fatty acids catalysed by lipoxygenase (Levavasseur et al., 2006; Nicolas & Drapron, 1983). Oxygen as electron acceptor can also be used by exogenous enzymes added as substitutes for chemical treatments of weak flours (Joye, Lagrain, & Delcour, 2009). Such addition was studied for carbohydrate oxidases such as glucose oxidase (GOX, EC 1.1.3.4) (Bonet, Rosell, Pérez-Munuera, & Hernando, 2007), hexose oxidase (HOX, EC 1.1.3.5) (Gül, Özer, & Dizlek, 2009; Poulsen & Høstrup, 1998) or pyranose oxidase (P2O, EC 1.1.3.10) (Decamps et al., 2012, 2013; Decamps, Joye, Courtin, & Delcour, 2012).

A carbohydrate oxidase purified from *Microdochium nivale* (COXMn) (Kulys, Tetianec, & Schneider, 2001; Xu et al., 2001) was characterised as a flavoenzyme, which catalyses both the oxidation of glucose and other mono- and oligosaccharides by reacting with the aldehyde function at C1, and the concomitant reduction of oxygen to hydrogen peroxide. The mechanism of the C1 oxidation by oxygen is a Ping Pong mechanism (Kulys et al., 2001). This oxidase can be considered as safe for use in human food (Ahmad, Brinch, Friis, & Pedersen, 2004). COXMn can also be used in biosensors for glucose determination in blood (Kulys, Tetianec, & Schneider, 2000), in the catalysis of the biotransformation of lactose into the value-added product lactobionic acid (Nordkvist, Nielsen, & Villadsen, 2007), in the generation of bleaching agents in liquid detergents (Pricelius, Ludwig, Lant, Haltrich, & Guebitz, 2011) and as a dough or bread improver (Arnaut, De Meyer, & Van Haesendonck, 2006; Christensen et al., 1999; Oestergaard & De Maria, 2011).

COXMn could be used as a substitute for oxidative compounds in bread making. Firstly, contrary to GOX, COXMn is able to catalyse the oxidation of a broad range of carbohydrates (Christensen et al., 1999; Kulys et al., 2001), especially the mono- or oligosaccharides found in wheat flour (Belitz, Grosch, & Schieberle, 2004; Potus, Poiffait, & Drapron, 1994; Souci, Fachmann, & Kraut, 2008). Hydrogen peroxide produced by reduction of O_2 would be used by endogenous wheat flour peroxidases to catalyse the oxidation of endogenous phenolic acids such as ferulic acid (Sosulski, Krygier, & Hogge, 1982) or tyrosine, forming homo- or heterodimers. This enzyme, similarly to GOX (Garcia, Rakotozafy, & Nicolas, 2004) and P2O (Decamps et al., 2012, 2012, 2013), can then promote crosslinking between pentosans and proteins (Tilley et al., 2001). Moreover, the addition of carbohydrate oxidases may be of great interest in the gluten and

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arabinoxylans network formation during dough mixing regarding the activity of endogenous enzymes affected by mixing such as catalase (Nicolas & Drapron, 1983; Brijs et al., 2009, chap. 11) and lipoxygenase (Delcros et al., 1998).

The purpose of this work was to study the activity of COXMn in the presence of ingredients and additives present in a wheat flour dough (pH activity, affinity towards oxygen and carbohydrates, adverse effect of NaCl and ferulic acid) in comparison to GOX. This gives insight into the suitability of COXMn as a possible flour and dough improver.

2. Materials and methods

2.1. Materials

M. nivale carbohydrate oxidase (COXMn) and *Aspergillus niger* glucose oxidase (GOX) were supplied as powders by Novozymes A/S. A suspension of COXMn in distilled water (32 mg mL^{-1}) was stirred 15 min and centrifuged at 1800 g during 5 min at 4 °C. The supernatant was used as the enzyme extract. A solution of GOX (0.75 mg mL^{-1}) was prepared in distilled water. Typical assay solutions contained 20 μL of the solution of COXMn (0.64 mg) or 30 μL of the solution of GOX (0.023 mg). These quantities provided the same activity (oxygen uptake) towards an air-saturated solution of glucose (0.22 mol L^{-1}) at 30 °C and pH 5.6.

D-Glucose, D-maltose, D-xylose, D-fructose, D-galactose, L-arabinose, raffinose, D-sucrose, maltotriose, maltotetraose and D-glucono-δ-lactone were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade from V.W.R. (Fontenay-sous-Bois, France).

The wheat flour used in this work was an untreated, improver-free straight-grade flour, commercially milled by Les Moulins Soufflet (Nogent-sur-Seine, France). Maize starch (Maïzena) was a commercial product from Alsa Unilever France (Rueil-Malmaison, France). Water soluble pentosans from wheat (around 0.1% (w/w) of ferulic acid, 21.9% (w/w) of xylose, 19.4% (w/w) of arabinose) were kindly provided by Luc Saulnier (INRA Biopolymers Interaction Assemblies, Nantes, France).

2.2. Preparation of aqueous extracts of wheat flour, wheat pentosans and maize starch

Wheat flour (4 g), maize starch (4 g) and pentosans (200 mg) were suspended in 10 mL of sodium acetate buffer (0.1 mol L^{-1}, pH 5.6). The suspensions were vortexed during 10 s, homogenised with an Ultraturrax T25 (IKA, Staufen, Germany) in an ice bath (15 s at maximal speed twice, followed by 15 s of rest) and centrifuged at 4 °C and 28,000 g for 20 min. The aqueous extracts were the supernatants of the suspensions after centrifugation. The carbohydrate contents of these aqueous extracts (fructose, glucose, sucrose, maltose, raffinose) were measured using the chromatographic method described by Potus et al. (1994).

2.3. COXMn and GOX oxidase activities

All oxidase activities were measured by polarography using an OSD 23 Oximeter (Heito, Paris, France) fitted with a Clark-type electrode placed in a thermostated cell. Activity was calculated from the initial rate of O_2 consumption and expressed in nkat (nmoles of consumed O_2 s^{-1}). Aliquots of enzyme solutions (20 μL for COXMn and 30 μL for GOX respectively) were added to carbohydrate solutions preincubated at 30 °C. Except for the true kinetic constants, all assays were done under air saturation corresponding to a molecular oxygen concentration of 0.25 mmol L^{-1}. Assays were made in sodium acetate buffer (0.1 mol L^{-1}, pH 5.6), except for the determination of the optimum pH range. The total reaction volume was 1.5 mL.

The effects of the pH on the COXMn activity were studied by screening the activity at various pH values (pH 3–8) towards air-saturated solutions of glucose (0.22 mol L^{-1}). Experiments were performed in buffers of sodium citrate–phosphate (0.1 mol L^{-1}, pH 3.1), sodium acetate (0.1 mol L^{-1}, pH between 4.0 and 5.6) and sodium phosphate (0.1 mol L^{-1}, pH between 5.9 and 8.0).

D-Glucose, D-maltose, D-xylose, D-fructose, D-galactose, L-arabinose, raffinose, D-sucrose, maltotriose and maltotetraose were screened as possible substrates for COXMn. The apparent kinetic constants (Michaelis Menten constant K_{mapp}, maximal reaction velocity V_{mapp}) of COXMn towards D-glucose, D-maltose, D-xylose and D-galactose, and of GOX towards D-glucose were determined by varying the concentrations of carbohydrates (0.4–1500 mmol L^{-1}) in air-saturated solutions at 30 °C.

The true kinetic constants (Michaelis Menten constants K_m, maximal reaction velocity V_m) of COXMn and GOX were determined by varying the concentrations of O_2 and D-maltose for COXMn, or D-glucose for GOX. Different concentrations (2–50 mmol L^{-1}) of each of the carbohydrate substrates were tested in the presence of different concentrations of O_2 (20–580 μmol L^{-1}) at 30 °C. Solutions with different O_2 levels were prepared by sparging the reaction solutions with nitrogen, air and oxygen. The initial O_2 concentration was determined by polarography.

Two types of substrates were then used:

–*Model solutions:* five mixtures containing D-glucose, D-maltose, D-xylose and D-galactose were prepared at various concentrations selected according to the measured apparent K_{mapp} for each carbohydrate. Four mixtures contained a maximum concentration of one of these carbohydrates. The measured activity was expected to be mainly due to the activity towards the carbohydrate of maximum concentration. A fifth mixture was prepared in order to have two thirds of the measured activity due to the activity towards D-maltose and D-glucose and one third of the measured activity due to the activity towards D-xylose and D-galactose. The COXMn activity was measured in these air-saturated mixtures at 30 °C.

–*Aqueous extracts:* the previous aqueous extracts of wheat flour, pentosans and starch were considered as providers of carbohydrate substrates for COXMn and GOX. The oxidase activities were measured by adding aliquots of enzyme solutions directly into the air-saturated aqueous extracts at 30 °C.

2.4. Effects of D-glucono-δ-lactone, hydrogen peroxide, sodium chloride and ferulic acid on the oxidase activity

All solutions were prepared in sodium acetate buffer (0.1 mol L^{-1}, pH 5.6) and saturated by air at 30 °C.

D-Glucono-δ-lactone was tested as possible substrate for COXMn and GOX. The effects of D-gluconolactone on the activities of COXMn and GOX were studied by measuring the activity at three distinct concentrations of D-gluconolactone (0, 0.25 and 220 mmol L^{-1}) in the systems: enzyme + D-glucose (20 and 220 mmol L^{-1}).

Tests were performed by incubating COXMn (91.5 mg) with hydrogen peroxide (21.45 mmol) for 4 h at room temperature in a total volume of 5 mL. The COXMn activity was measured regularly by injecting 35 μL of this mixture into a 0.3 mol L^{-1} D-glucose solution.

The effects of NaCl on the activities of COXMn and GOX were studied by measuring the activity at a concentration of 0.39 mol L^{-1} in the systems: COXMn + D-maltose and GOX + D-glucose. The carbohydrate concentrations were varied