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Critical assessment of the formation of hydrogen peroxide in dough by fermenting yeast cells

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ABSTRACT

Fermentation of bread dough leads to strengthening of the dough matrix. This effect has previously been ascribed to the action of hydrogen peroxide (H_2O_2) produced by yeast in dough. In this study, we re-evaluate the production of H_2O_2 by yeast in dough and aqueous fermentation broth. Results show that the previously reported high levels of H_2O_2 in fermenting dough were most probably due to the lack of specificity of the potassium dichromate/acetic acid-based method used. Using the chemiluminescent HyPerBlu assay, no yeast H_2O_2 production could be detected in fermented dough or broth. Even though the formation of low levels of H_2O_2 cannot be ruled out due to the presence of catalase in flour and the fast reaction of H_2O_2 with gluten proteins, our results suggest that the changes in dough matrix rheological properties upon fermentation are not due to production of H_2O_2 by yeast.

1. Introduction

The common baker's yeast *Saccharomyces cerevisiae* is the main leavening agent in bread making and is responsible for the typical aerated structure of leavened bread. Interestingly, apart from the leavening, fermentation also has a significant impact on the rheology of the dough matrix (Hoseney, Hsu, & Junge, 1979; Jayaram et al., 2013; Nagao, Endo, & Tanaka, 1981; Newberry, Phan-Thien, Larroque, Tanner, & Larsen, 2002). Specifically, the effect of fermentation on dough rheology has been compared to oxidation (Hoseney et al., 1979; Nagao et al., 1981; Newberry et al., 2002) and has been suggested to be caused by the release of hydrogen peroxide (H_2O_2) by the fermenting yeast cells (Liao, Miller, & Hoseney, 1998).

Much like other chemical oxidants, H_2O_2 strengthens the gluten network and makes the dough more tolerant to over-mixing (Bekes, Gras, & Gupta, 1994; Dunnewind, van Vliet, & Orsel, 2002; Takasaki, Kato, Murata, Homma, & Kawakishi, 2005). Addition of H_2O_2 increases dough elasticity, decreases dough extensibility and the use of optimal concentrations improves overall bread quality (Liao et al., 1998). Oxidising enzymes, such as glucose oxidase and pyranose oxidase, have also been suggested to increase dough stability and bread loaf volume by producing H_2O_2 (Decamps, Joye, Courtin, & Delcour, 2012; Joye, Lagrain, & Delcour, 2009a).

Different mechanisms have been proposed for the strengthening effect of H_2O_2 on dough rheology. H_2O_2 is suggested to reinforce the gluten network through the oxidation of cysteine residues and formation of disulphide bonds (Bonet et al., 2006). Furthermore, H_2O_2 could stimulate the formation of dityrosine cross-links through oxidative coupling of tyrosine residues in gluten proteins (Tilley et al., 2001). These changes in the degree of crosslinking of gluten proteins can improve handling and machinability of dough and also reduce dough stickiness (Decamps et al., 2012; Joye, Lagrain, & Delcour, 2009b). H_2O_2 might also cause oxidative cross-linking of ferulic acid residues in water-extractable arabinoxylans, through the action of wheat flour peroxidase, which could increase the viscosity of the aqueous phase in dough (Courtin & Delcour, 2002; Schofield, 1996). Moreover, it has been suggested that ferulic acid residues in arabinoxylan can cross-link with tyrosine residues in gluten proteins through the same mechanism. However, these cross-links are scarce and they may therefore not have a significant effect on the functional properties of dough (Piber & Koehler, 2005).

Abbreviations: RLU, Relative Light Units; YPD, yeast peptone dextrose; YPS, yeast peptone sucrose; OD_{600}, optical density at 600 nm.

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H_2O_2 is one of the major reactive oxygen species in living cells, where it is generated as a by-product of oxidative metabolism (Bienert, Schjoerring, & Jahn, 2006; Jamieson, 1998; Sousa-Lopes, Antunes, Cyrne, & Marinho, 2004; Zhou, Wang, Guo, Tan, & Zhu, 2006). It was previously believed that the H_2O_2 produced in yeast mitochondria could freely cross the cell membranes and diffuse into the surrounding medium (Boveris, 1978; Boveris & Chance, 1973; Liao et al., 1998). However, more recent reports demonstrated that biomembranes show poor permeability towards H_2O_2, suggesting that H_2O_2 transport does not depend on passive diffusion (Bienert et al., 2006). *S. cerevisiae* cells seem able to regulated the membrane permeability towards H_2O_2 by controlling the activity of certain aquaporins that function as facilitators for H_2O_2 transport, a mechanism that has also been observed in plant and mammalian cells (Bienert et al., 2007; Dynowski, Schaaf, Loque, Moran, & Ludewig, 2008; Hooijmaijers et al., 2012; Miller, Dickinson, & Chang, 2010).

The objective of this study was to reevaluate the production of H_2O_2 by yeast in dough and aqueous fermentation broth. We show that the previously used method to measure H_2O_2 that was based on potassium dichromate/acetic acid is inaccurate in dough. Instead, a more recent method based on chemiluminescent detection of H_2O_2 using the HyPerBlu substrate shows that there is no or very little H_2O_2 in dough, suggesting that other metabolites, such as organic acids and ethanol, are responsible for changing the dough matrix rheology during fermentation.

2. Materials and methods

2.1. Materials

A commercial wheat flour, free of additives, was obtained from Ceres-Soufflet (Brussels, Belgium). Wheat gluten and starch were obtained from Tereos Syral (Aalst, Belgium) and used to make a gluten–starch blend containing 16% gluten and 84% starch. A commercial *S. cerevisiae* strain (V1116) was provided by VIB Laboratory of Systems Biology (KU Leuven, Belgium). Yeast extract and balanced peptone were procured from Lab M (Brussels, Belgium). All other chemicals, solvents and reagents were purchased from Sigma–Aldrich (Bornem, Belgium) and are of analytical grade unless specified otherwise.

2.2. Flour characterisation

Moisture content was measured according to AACC method 44–19.01 (AACC, 2000). Protein content (on dry matter base) was determined according to AOAC method 990.03 (AOAC, 1995), which was adapted for an automated Dumas protein analysis system (EAS, VarioMax N/CN, Elt, Gouda, The Netherlands). All measurements were carried out in triplicate. The commercial wheat flour and the gluten–starch blend had a moisture content of 14.0% and 10.4%, respectively, and a protein content of 10.5% and 13.3%, respectively.

2.3. Spectrophotometric measurement of H_2O_2 using potassium dichromate/acetic acid

The colorimetric assay for H_2O_2 measurement using a potassium dichromate/acetic acid reagent was initially introduced by Sinha (1972). It is based on the reaction of potassium dichromate in acetic acid with H_2O_2, resulting in the formation of perchromic acid as blue precipitate. This unstable intermediate is then reduced to chromic acetate (green) upon heating, which can be measured spectrophotometrically.

Dough extract (1.0 mL) was mixed with potassium dichromate in acetic acid solution (1:3, v/v) (2.0 mL) and the mixture was heated to 80 °C in a water bath (GFL model 1003, Burgwedel, Germany) for 10 min. The sample was cooled to room temperature and the absorbance of the reaction mixture was measured at 570 nm using an Ultraspec 2000 spectrophotometer (GE Healthcare, Diegem, Belgium). A calibration curve, prepared using H_2O_2 concentrations ranging from 17 to 4250 μmol/100 g flour, was used to calculate the H_2O_2 concentration in the dough extracts.

2.4. Chemiluminescent measurement of H_2O_2 using HyPerBlu

The chemiluminescent assay for H_2O_2 measurement uses the HyPerBlu substrate (a dioxetaneboronic acid) (Lumigen, Michigan, USA) for direct chemiluminescent detection of H_2O_2. HyPerBlu reacts with H_2O_2 in alkaline conditions and forms an unstable intermediate that readily dissociates and gives rise to a strong luminescent signal (Fig. 1). The intensity of this luminescent signal can be detected using a microplate reading luminometer with a photo multiplier tube detector (Coulter, 2011).

Measurements were performed in a 384-well white microtitre plate (Thermo Scientific, Cheshire, UK) in which 5.0 μL sample and 5.0 μL tris(hydroxymethyl)aminomethane (Tris) HCl buffer (0.3 M, pH 9.0) were mixed. The reaction was started by adding 5.0 μL of Lumigen HyPerBlu substrate to the sample. After 25 min incubation in the dark at room temperature, the luminescence intensity was measured using a Synergy Mx plate reader (Biotek, Winooski, USA). A calibration curve, made with H_2O_2 concentrations ranging from 0.1 to 4.2 μmol/100 g flour was used to calculate the H_2O_2 concentration in the dough extracts. All measurements were performed in triplicate on two different doughs.

2.5. Preparation and growth of yeast

Yeast cells from a glycerol stock were grown on yeast peptone dextrose (YPD) agar plates with the following composition: 1.0% w/v yeast extract, 2.0% w/v balanced peptone, 2.0% w/v dextrose and 2.0% w/v agar. Subsequently, they were transferred to yeast peptone sucrose (YPS) medium, a solution of 1.0% w/v yeast extract, 2.0% w/v balanced peptone and 2.0% w/v sucrose, for further propagation of the cells. The above-mentioned agar and YP medium were both sterilised by autoclaving (Timo bench top autoclave, Pbi International, Milan, Italy). Dextrose solution (20.0% w/v)

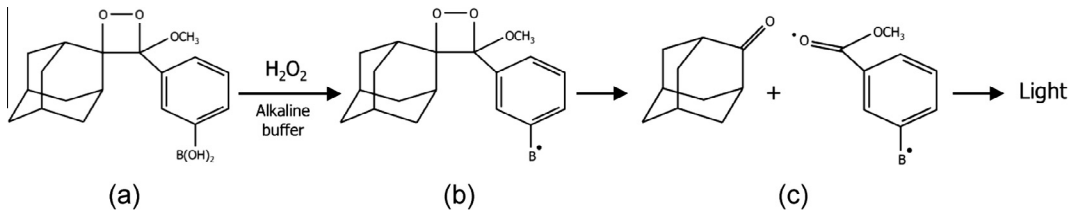

Fig. 1. Reaction of Lumigen HyPerBlu (a) with H_2O_2 gives rise to an unstable dioxetane intermediate (b) that readily dissociates (c) resulting in an intense luminescent signal (Coulter, 2011).