CHEMICAL ENGINEERING TRANSACTIONS
VOL. 87, 2021
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.cetjournal.it
Guest Editors: Laura Piazza, Mauro Moresi, Francesco Donsì

ISBN 978-88-95608-85-3; **ISSN** 2283-9216

Regulation of Peroxidase Activity in Flour Using Supercritical Fluids

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Enzymes present in flour have crucial function in fermentation process, where some of them are important from the standpoint of baking. Moreover, flour naturally contains several technologically important enzymes such as amylases, proteases, lipoxygenase, polyphenol oxidase and peroxidase. Since these enzymes are an important factor, which affect the quality of flour already during storage and particularly in the manufacturing process of the final bakery product, is this contribution focused on the activity of peroxidase enzyme in graham flour and the importance of this enzyme during baking process. Enzyme peroxidase catalyzes the conversion of a peroxide such as hydrogen peroxide into its basic constituents, such as H_2O_2 into H_2O and O_2. Moreover, peroxidase is responsible for disliked color changes in food, therefore, it is important to regulate the enzyme activity, with which the quality of the product should maintain and thus the storage time of the flour will be extended.
Furthermore, residual enzyme activity was determined with activity assay using a UV-spectrophotometer, after the exposure of the flour under supercritical conditions in high pressure batch reactor. Supercritical carbon dioxide was used as an environmentally friendly solvent, which reaches supercritical conditions even at low operational temperatures (35 °C). The present study compares between initial and residual activities of peroxidase and shows the influence of supercritical CO_2 on peroxidase activity in graham flour.

1. Introduction

Graham flour is a form of whole wheat flour made by grinding the endosperm and is thus also the most nutritious. Compared to other types of flour, graham flour contains more fiber, minerals, vitamins, and bioactive compounds (Gómez et al., 2020), such as proteins and enzymes.
The purpose of the study was the inactivation of enzymes in flour after exposure to supercritical carbon dioxide ($scCO_2$). $ScCO_2$ is the most studied supercritical fluid (SCF) today because of its economic, technical, environmental benefits and thus suitable for treatment of food. In addition, it is an excellent solvent for the transport of hydrophobic compounds. Also, the critical pressure is relatively low (7.34 MPa) and has a low critical temperature (31 °C), which provides the mild conditions required to maintain enzyme activity (Hu et al., 2013; Zhong & Jin, 2008). Also, carbon dioxide is at the maximum oxidation number of carbon and therefore is inert towards further oxidation (i.e., non-flammable). The conditions selected reflect those that would be considered in the design of the industrial process.
Peroxidase enzymes (EC 1.11.1.7) catalyze the oxidation of several substrates in the presence of hydrogen peroxide and are of particular interest to cereal scientists. During wheat kernel development, peroxidase contributes to restricting seed elongation. In breadmaking, arabinoxylans in wheat and rye undergo oxidative coupling of ferulic acid residues, causing an increase in dough viscosity on mixing in the presence of peroxidase and hydrogen peroxide. Peroxidase also plays a significant role in carotenoid bleaching during dough mixing and may be responsible for undesirable changes, including deterioration of color and flavor (Hatcher & Barker,

2005). According to that, Suzuki et al., (2005) examined which enzymes (lipase, lipoxygenase, or peroxidase) are important for quality deterioration in buckwheat flour, where the effects of changes in enzymes activities were investigated (Suzuki et al., 2005).
Enzyme inactivation is generally explained as a chemical process involving several phenomena like aggregation, dissociation into subunits, or denaturation (conformational changes), which occur simultaneously during the inactivation of a specific enzyme (Polakovi• et al., 1998). The enzyme inactivation mechanism most probably include disruptive changes in the active site, blockage of substrate access, unfavourable substrate desolvation and effects of transition state destabilization and restriction of conformational mobility. The three-dimensional structure of enzymes is dramatically altered under extremely dehydrating conditions, causing their denaturation and a consequent loss of their activity. However, if the conditions are less adverse, the protein structure may largely be retained. For example, $scCO_2$ may dissolve from 0.3% to 0.5% (w/w) water, depending on the pressure and temperature (Budisa & Schulze-Makuch, 2014).

The methods for enzyme inactivation in the vast majority apply heat treatment. In general, heat treatment often deteriorates food properties, many chemical agents are not compatible with food products due to their toxicity or adverse effects on sensory properties, and gamma radiation is not accepted by consumers (Butscher et al., 2020). To solve this challenge, $scCO_2$ treatment has been proposed as a promising alternative for enzyme inactivation in foods and food products. The contribution describes inactivation of enzyme peroxidase in graham flour, where the activity of peroxidase is affected by $scCO_2$ treatment under different conditions.

2. Materials and methods

2.1 Materials

Graham flour was kindly donated by the bakery Hleb• ek d.o.o. (Pragersko, Slovenia). Analyses were conducted using carbon dioxide (99.5% purity) produced by Messer, Ruše. Ethanol, phosphoric acid, Coomassie-Brilliant Blue G250 and phenol were supplied from Merck (Germany), while bovine serum albumin (BSA), sodium acetate, 4-aminoantipyrine and hydrogen peroxide were supplied from Sigma (Germany). Potassium dihydrogen phosphate (KH_2PO_4) were purchased from Acros Organics (Nidderau, Germany). All other chemicals used in the laboratory were of analytical grade.

2.2 Methods

2.2.1 Supercritical CO_2 treatment

Graham flour was exposed to $scCO_2$ in a 60 mL high-pressure batch reactor at 35 °C to the desired pressure for a defined time, with the $scCO_2$ system shown in Figure 1.

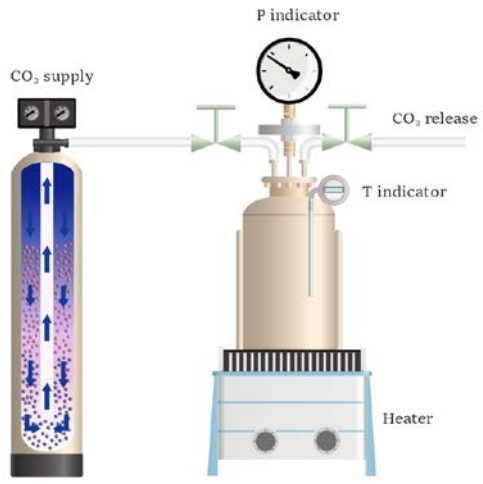

Figure 1: Supercritical carbon dioxide reactor scheme.

When the temperature in the high-pressure batch reactor with graham flour reached 35 °C, cooled CO_2 was added to the reactor until the desired pressure was obtained. Afterwards, the reactor was quickly depressurized. Furthermore, a sample of flour was taken out of the reactor and left at room temperature for some time to ensure that CO_2 was released out of the sample, which was described in detail in our previous paper (Hojnik Podrepšek et al., 2020a). The enzyme activity of the treated samples of graham flour was compared with that of the untreated graham flour.

2.2.2 Extraction of flour proteins

Proteins were extracted from graham flour (5 g) with 0.1M acetate buffer solution (pH 5.3) by shaking for 90 min at room temperature at 300 rpm on laboratory horizontal shaker (New Brunswick, Excella® E24/E24R). To ensure efficient disruption, homogenization and higher concentration of protein from flour, glass beads were added to the mixture. The suspension was then centrifuged at a speed of 8000 rpm for 5 min, and the clear supernatant was collected.

2.2.2 Determination of protein concentration

Protein concentrations were determined in supernatant samples using the Bradford spectrophotometric method. Measurements were carried out using a UV-Vis spectrophotometer (Varian Cary Probe 50, Agilent Technologies, Santa Clara, United States) at a wavelength of 595 nm. The calibration curve was constructed using bovine serum albumin (BSA) as a standard. Moreover, the protein concentration *c* (mg/mL) in the samples was calculated using the standard curve of BSA from Eq. (1),

$$c = \frac{A_{595\,nm}}{k}\ (\frac{mg}{mL}) \tag{1}$$

where $A_{595\,nm}$ is the sample absorbance measured at 595 nm, and *k* is the slope of the calibration curve, according to Bradford method (Bradford, 1976).

2.2.3 Enzyme Activity Measurements

Enzyme activity of peroxidase was evaluated by measuring the absorbance of the colorimetric assay using UV-Vis spectrophotometer (Varian Cary Probe 50, Agilent Technologies, United States). The residual activity of peroxidase in untreated and scCO_2-treated graham flour was determined by an activity assay based on the oxidation reaction of 4-aminoantipyrine (4-AAP) in the presence of hydrogen peroxide. Phenol, 4-AAP, and H_2O_2 were used as color-generating substrates. The reaction rate was determined by measuring an increase in absorbance at 510 nm resulting from the decomposition of hydrogen peroxide. A mixture of 1.5 mL H_2O_2 solution (1.7 mM in PBS) and 1.4 mL phenol solution (containing 0.17 M phenol and 2.5 mM 4-aminoantipyrine in distilled water) was prepared and enzyme solution was added to the mixture; the absorbance at 510 nm was monitored for 5 min in the open cuvette following the initiation of the reaction. One unit of specific activity was defined as the consumption of 1 µmol H_2O_2 per milligram of enzyme in 1 min at 25 °C at pH 7.0, calculated according to Equation (2) from (Hojnik Podrepšek et al., 2020b)

$$Enzyme\ activity\ (\frac{U}{mg}) = \frac{\Delta A/min}{(\varepsilon * m_e) * V_s} \tag{2}$$

where *U/mg* is the specific activity of the peroxidase enzyme (mol·mg^{-1}·min^{-1}), • *A/min* is the slope of the 4-AAP oxidation reaction curve (min^{-1}), μ is the molar extinction coefficient (6.58 M^{-1}·cm^{-1}), m_e is the enzyme mass in the sample (mg) and V_s is the volume of the reaction solution (mL). Determination of protein concentration and peroxidase activity in graham flour samples were repeated three times to obtain reliable results, within an experimental error about 0.5%.

3. Results

This study was conducted to evaluate the effects of $scCO_2$ treatment on protein concentration and peroxidase activity in graham flour.

3.1. The influence of $scCO_2$ on protein concentration and peroxidase activity

The protein concentration after protein extraction from graham flour was determined by the Bradford method. The effect of $scCO_2$ on protein concentration was evaluated after graham flour exposure to $scCO_2$ at different conditions, with the results presented in Figure 2. Furthermore, the protein concentration in untreated flour was taken for comparison as an initial value. Figure 2 presents the protein concentration in untreated and $scCO_2$-treated graham flour. As can be seen from the results, the protein concentration in graham flour decreased after $scCO_2$ exposure. Additionally, with increasing pressure it is observed that the protein concentration was reduced by 19 % after $scCO_2$ treatment for 24 h and 200 bar, confirming that $scCO_2$ induced protein loss in graham flour. Protein concentration is further reduced by exposure to $scCO_2$ and treatment time 48 hours to 78 %. The protein loss in $scCO_2$-treated graham flour in this contribution is mainly ascribed to leakage of proteins into the media.

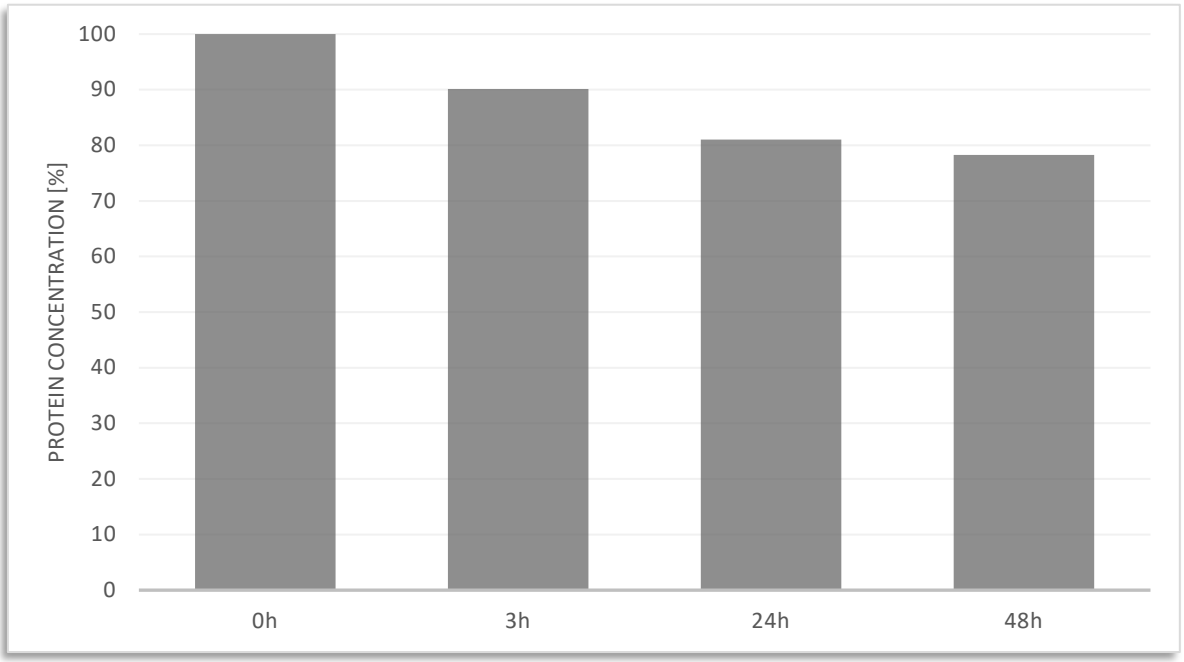

Figure 2: Protein concentration of untreated (0h) and $scCO_2$-treated graham flour at 200 bar after different treatment times.

The influence of $scCO_2$ on the residual activity of the peroxidase enzyme in graham flour at different pressures and with different treatment times at 35 °C is presented in Figure 3. The results show that peroxidase activity decreased with an increase of treatment pressure. A 20 % decrease in peroxidase activity occurred after 3 h at 200 bar, and the residual activity of peroxidase after 24 h of $scCO_2$ treatment of graham flour at 200 bar was 74 %. Additionally, the graham flour exposure in $scCO_2$ for 48 hours resulted in a further decrease in peroxidase activity, which decreased to 73%. Taken together, these data clearly demonstrate that similar to the results for protein concentration, at a higher pressure a greater inactivation of the peroxidase activity in graham flour was achieved.

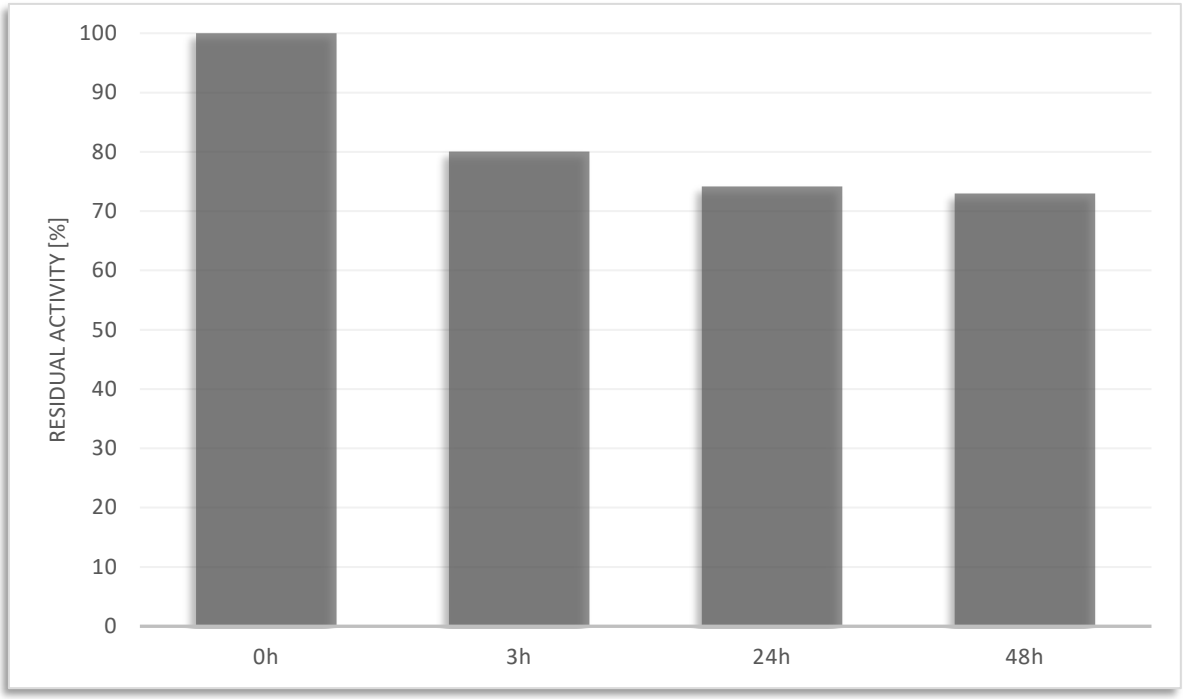

Figure 3: Residual activity of peroxidase of untreated (0h) and $scCO_2$-treated graham flour at 200 bar after treatment times of 3 and 24 hours.

Although the mechanism of enzyme inactivation under $scCO_2$ has not been fully determined, the potential mechanisms suggest the important effect of intramolecular interactions, the hydration of charged groups, disruption of bound water, and stabilization of hydrogen bonds (Eisenmenger & Reyes-De-Corcuera, 2009). Marszałek et al., (2019) extensively discussed that the hydrogen bond may be strengthened slightly under pressurization, because of the decrease in the inter-atomic distance leading to a smaller molecular size. The impact of pressure on intra- and intermolecular interactions within enzymes could also be connected with the ability of the enzymes´ functional groups to interact with water. The promotion of charged dipoles in an aqueous medium is initiated by high pressure because the electrostriction of water around the charges decreases the molar volume of water. This means that an increase in pressure will weaken the electrostatic or coulombic interactions (Marszałek, Doesburg, et al., 2019). Enzyme inactivation using $scCO_2$ is a very complex phenomenon. This mechanism involves a series of physical changes such as the formation and/or disruption of numerous bond interactions as well as changes in the structure of the enzymes by folding and/or unfolding proteins during pressurization and depressurization (Chakraborty et al., 2014). Certainly, the effect of $scCO_2$ techniques can be described by the changes in the tertiary and even secondary structure of proteins, thus leading to active site distortion and the formation of new bonds, which consequently decreases the concentration of free ions (Marszałek, Szczepańska, et al., 2019).

4. Conclusions

The aim of this study was to investigate the effects of $scCO_2$ on the protein concentration and inactivation of enzyme peroxidase in graham flour after $scCO_2$ exposure. $ScCO_2$ treatment had significant effects on the residual activity of peroxidase. Moreover, assay for determination of peroxidase activity in flour based on decomposition of hydrogen peroxide, was shown to be successful, because during the study the peroxidase activities in untreated graham flour and $scCO_2$-treated graham flour were properly determined. Afterwards, $scCO_2$ treatment achieved peroxidase inactivation with residual activity of 72 % after a 48 h treatment at 200 bar. Therefore, the non-thermal $scCO_2$ treatment is a promising technology since it is recognized as an environmentally friendly technology and the development of new processes has been encouraged in many industrial sectors and research fields.

Acknowledgments

The authors acknowledge the Slovenian Research Agency and Ministry of Education, Science, and Sport, Slovenia. We appreciate the help of Metka Slomaek and Hleb• ek d.o.o., Pragersko, Slovenia for the donation of flour samples and Domen Petri• for technical support.
Prof. Dr. Maja Leitgeb and Prof. Dr. Željko Knez are partly employed at University of Maribor, Faculty of Medicine, Maribor, Slovenia.

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