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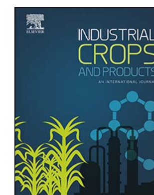

Controlled enzymatic hydrolysis for improved exploitation of the antioxidant potential of wheat gluten

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ABSTRACT

The aim of the study was to find the optimal operational and process parameters for the enzymatic hydrolysis of wheat gluten in a batch stirred bioreactor regarding both degree of hydrolysis and antioxidant capacity of the obtained hydrolysates. It appeared that impeller geometry and agitation speed influenced the mass transfer resulting in enhanced gluten hydrolysis. The highest initial reaction rate ($0.83 \pm 0.02\ min^{-1}$) and degree of hydrolysis (30.47%) were achieved with the pitched four-bladed impeller and agitation speed of 350–450 rpm, conditions which provided proper balance between requirements for adequate mass/heat transfer and low shear stress. The impact of other process conditions including gluten concentration, temperature, pH and enzyme-gluten (*E/S*) ratio on the enzymatic reaction was investigated by applying a Box-Behnken experimental design from the viewpoint of the degree of hydrolysis (DH) and antioxidant activity. Three models obtained allowed calculation of the hydrolysis degree, and both 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulpfonic acid) radical scavenging activity from a given set of reaction conditions with good predictability. The statistical analysis showed that each variable had a significant effect on degree of hydrolysis and the antioxidant capacity of both tested systems. Hydrolysis up to around DH 15% improved DPPH radical scavenging activity, while excessive hydrolysis worsened it. The ABTS activity of the hydrolysates was not associated with the DPPH activity nor with the DH, revealing that it was not possible to fulfill all desirable quality requirements (maximum degree of hydrolysis and protein yield, maximum DPPH and ABTS scavenging activities) by using the same reaction conditions. Overall, the study might contribute to approve wheat gluten, a by-product of wheat starch industry, as an accessible and cheap source of bioactive compounds for the development of novel nutraceuticals, cosmetics and drugs.

1. Introduction

The use of low grade and renewable starting material including agricultural crops for the new bioactive ingredient production is still a significant challenge. Gluten, a by-product of wheat starch industry, is a promising source of valuable functional ingredients in the form of soluble protein hydrolysates due to its natural origin, availability, low cost and health-related benefits associated with their hydrolysates (Matsui et al., 2000; Kong et al., 2008). Wheat gluten is a rather complex protein composed of two seed storage proteins, gliadins and glutenins. Glutenins, the major proteins of flour, are poorly soluble in alcohols because they are capable to form large polymers that are stabilized by intermolecular disulfide bonds and hydrophobic interactions (Koehler and Wieser, 2013). In contrast to glutenins, gliadins are soluble in aqueous alcohol (for example 60–70% ethanol) and are mainly present in gluten as monomers interacting by non-covalent forces. Native wheat gluten is usually used to texture wheat breads and foods having unique functional properties during bread making. Recent research has also shown that wheat gluten and its hydrolysates, as the most complex protein and peptides, have higher concentration of disulfide bonds (45.37 nmol/mg) and total cysteine (93.88 nmol/mg) compared to other tested proteins (maize proteins, pea protein isolates), showing the highest ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulpfonic acid) radical scavenging activity (74.39 mmol Trolox/kg). The antioxidant capacity is positively correlated with total cysteine and −S−S bond concentration indicating a high effect of physical structure

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of a protein on its antioxidant properties (Žilić et al., 2012).

Despite the low cost and unique viscoelastic properties of gluten proteins, they are still often exploited in low cost applications such as animal feed (Day et al., 2006). The lack of functional properties such as emulsifying properties or solubility, especially close to its isoelectric point at pH 6–7 may limit the use of native wheat gluten in many other applications such as nutraceuticals, cosmetics and drugs. Previous studies have shown that the controlled and mild enzymatic hydrolysis could be successfully applied to improve and upgrade the functional properties of gluten (Kong et al., 2007; Koo et al., 2014). It is also an efficient tool for the production of low molecular peptides with antioxidant properties from gluten protein, including the capacity to inhibit the linoleic acid peroxidation or to quench the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical (Kong et al., 2008; Suetsuna and Chen, 2002; Wang et al., 2006;). In this case, processing conditions, specificity of the proteolytic enzymes used, and the degree of hydrolysis also greatly affect the antioxidant properties of the obtained hydrolysate and should be controlled.

The design and optimization of the protein hydrolysis in the batch reactor system is a rather difficult task due to the unfavorable reaction equilibrium, product inhibition, and enzyme inactivation to the enzyme autolysis and/or hydrodynamic stresses. Especially, the system tested in this study is very complicated, since gluten dispersions are highly viscous and exhibit non-Newtonian flow properties (Song and Zheng, 2009). This creates problems with both mass and heat transfer within the batch reactor as well as mechanical problems related to pumping and efficient mixing of the reaction mixture. The selection of impeller types and configurations and the understanding of the performance of mixing are, thus, important parts of the process design. Regarding generally protein hydrolysis, mixing in the batch reactor is achieved by orbital shaking or by means of hotplate stirrer and only little information on the effect of mechanical agitation on reaction rate and productivity is available (Jakovetić et al., 2015; Nouri et al., 1997).

Rational design of process parameters could lead to improved protein yield and adjusted properties, such as solubility, and antioxidant capacity (Deng et al., 2016; Ng et al., 2013; Valdez-Flores et al., 2016). In spite of the industrial importance of the gluten as a multifunctional ingredient of food/cosmetic interest, very little is found in the literature about the production of peptides with antioxidant activities from wheat gluten and the relationship between operating conditions, degree of hydrolysis and antioxidant capacity. Functional and biological properties of corn gluten hydrolysates has been intensively studied recently with particular emphasis on *in vivo* and *in vitro* antioxidant activity (Jin et al., 2016; Wang et al., 2016), but little is known about the biological activity of enzymatic hydrolysates originating from wheat gluten (Cian et al., 2015).

This study was designed to examine how different factors such as impeller types and agitation speeds affected the enzymatic hydrolysis of wheat gluten protein in terms of the achieved DH and reactor productivities. Further, the relationship between process parameters and antioxidant properties of the obtained hydrolysates, measured by two methods, was studied using Box-Benken experimental design and response surface methodology (RSM). The hydrolysate showing the highest DPPH· and ABTS$^{\cdot +}$ radical quenching ability, was further separated by sequential ultrafiltration and antioxidant properties of the different peptide fractions were also studied.

2. Materials and methods

2.1. Materials

Wheat gluten (moisture content: 6.8%, protein content (N 5.70): 78.52% on dry basis), was from MP Biomedicals (Santa Ana, CA). The commercial protease Alcalase® from *Bacillus licheniformis*, (EC 3.4.21.14) with the claimed activity of 2.4 Anson unit (AU)/g was used for enzymatic hydrolysis. The protease and other chemicals like bovine serum albumin (BSA), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma Aldrich (St. Louis, USA). The ultrafiltration (UF) stirred cell unit (model 8050 1 unit) and cellulose membranes with 30, 10 and 3 kDa molecular weight cut-off (MWCO) for the preparation and fractionation of hydrolysates was from Millipore Co. (Bedford, MA, USA). The deionised water (18.2 MΩ) used for the experiments was produced using a Thermo Scientific Barnstead Smart2Pure water purification system. All other chemicals used in this research were of analytical grade.

2.2. Bioreactor set up: apparatus and measurement

Enzymatic hydrolyses were conducted in a stirred tank reactor consisted of a 600 mL glass vessel with an inner diameter of 8 cm, flat bottom and at a working volume of 330 mL. The reactor was equipped with a water jacket, a pH electrode (Eutech instrument, Netherlands), heating unit (C-MAG HS 7, IKA, Germany) and an impeller-agitator (Heidolph RZR 2020, Germany). The pitched blade impeller with four blades (pitch angle 24°) was used unless otherwise indicated. The distance from the bottom wall was kept constant at 3.2 cm throughout the experiments. For selection of the most appropriate impeller type and configuration, four impeller geometries were considered and compared with each other; a single helical ribbon (diameter 75 mm, width 7.5 mm), anchor impeller (diameter 72 mm and a blade width 7.1 mm), four blades pitched impeller (with a diameter 68.2 mm and a blade width of 10 mm), and beater paddle (diameter 42.7 mm).

2.3. Preparation of gluten hydrolysates

An aqueous dispersion of wheat gluten (typically 5% w_{protein}/v) was stirred and pretreated by thermal treatment as previously described (Elmalimadi et al., 2017). After that, the obtained suspension was allowed to equilibrate at the working temperature (40–60 °C) for 20 min. Thereafter, the pH of the obtained suspension was adjusted to working level (7.0–9.0) with 0.8 M NaOH, and the hydrolysis was initiated by addition of the appropriated amount of alcalase (typically *E/S* was 0.50 AU/g of gluten protein). Enzymatic hydrolysis was carried out at constant pH, temperature and agitation (typically 200 rpm) while the progress of the reaction was followed using a pH stat method. The *DH* was calculated according Equation (Adler-Nissen, 1979):

$$DH\,(\%) = \frac{h\cdot 100}{h_{tot}} \tag{1}$$

where h represents the number of equivalents of peptide bonds hydrolyzed at the time expressed in meq/g; and h_{tot} is the theoretical amount of peptide bonds in the protein per weight unit of a protein (meq/g) and can be calculated from its amino acid composition (for wheat gluten protein h_{tot} is 8.38 mmol/g of protein) (Nielsen et al., 2001).

The h value was determined by pH stat technique, which is based on the assumption that the amount of peptide bonds cleaved is proportional to the amount of base consumed, and can be calculated using Eq. (2):

$$h = \frac{N_b B}{\alpha m_{\mathrm{p}}} \tag{2}$$

where B is the volume of base necessary to keep pH constant (mL), N_b is the normality of the base (meq/mL), α is the average degree of dissociation of the α-amino groups (0.88 at 50 °C and pH 8.0) and m_{p} is the mass of protein in g. The α value is pH dependent and can be estimated by Eq. (3):

$$\alpha = \frac{10^{p\mathrm{H}-pK}}{1 + 10^{p\mathrm{H}-pK}} \tag{3}$$

where *p*H is the value at which enzymatic hydrolysis is performed and

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