Food Control 110 (2020) 106987

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Prolyl endopeptidase from *Aspergillus niger* immobilized on a food-grade carrier for the production of gluten-reduced beer

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ARTICLE INFO

Keywords:
Prolyl endopeptidase
Covalent immobilization
Continuous treatment
Gluten reduction
Beer

ABSTRACT

The recently growing demand of gluten-reduced beer is leading to the development of diverse approaches to be applied in brewing. The current work focuses on the development of an innovative and sustainable biocatalytic tool for the continuous production of gluten-reduced beer, based on the application of immobilized prolyl endopeptidase from *Aspergillus niger* (AN-PEP). This food-grade protease has been immobilized on *A. niger* chitosan beads and applied, for the first time, for the reduction of gluten in a commercial beer from barley malt. The immobilization procedure was optimized for maximizing the specific activity of the biocatalyst (0.016 I.U./mg_{BSAeq}) and the best performance was reached using an immobilization solution at an initial protein concentration of 0.3 mg_{BSAeq}/mL.

The immobilization increased the thermal stability of the protease, which showed similar catalytic properties in synthetic beer (toward the synthetic substrate Z-Gly-Pro-pNA) when it was applied at 20 °C or at 50 °C. The continuous treatment in fluidized bed reactor (FBR), containing 10 g of immobilized AN-PEP (corresponding to 0.0036 g_{BSAeq}), was optimized varying the flow rate (Q_v). The suitable conditions to achieve reduction of the intact gluten of authentic beer was Q_v of 728 mL/min. The continuous treatment in FBR allowed us to reduce the initial gluten content (65 mg/kg) in the commercial beer from barley malt, reaching the concentration of 19 mg/kg after 9 h and 15 mg/kg after 10 h of treatment.

1. Introduction

Beer is an alcoholic beverage consumed throughout the world, with an average annual consumption of about 74 kg/capita in Europe and 86 kg/capita in Northern America (Colen & Swinnen, 2016). As reported by Barth-Haas (2013), the European beer market is second to the U.S. beer market, with a production volume of 545 million hectolitres. People affected by celiac disease and non-celiac gluten sensitivity represent about 6% of the global population (Carroccio et al., 2012; Genetics Home Reference, 2015). Individuals with celiac disease must adhere to a gluten-free (GF) diet. Therefore, they can safely drink beer surrogates which differ significantly from authentic (barley-based) beer in terms of aroma and taste. The Codex Alimentarius Standard and the EU-regulation 828/2014 established a gluten concentration below 20 mg/kg for GF food (Knorr, Wieser, & Koehler, 2016; Shan, 2002), whereas the range of gluten content in brewed barley malt beers may vary from < 10 mg/kg in some industrial lager beer to 4000 mg/kg in Weissbier (Fanari et al., 2017, 2018; Guerdrum & Bamforth, 2011; Hager, Taylor, Waters, & Arendt, 2014; Van Landschoot, 2011; Watson, Decloedt, Vanderputten, & Van Landschoot, 2018).

In the last decade, the demand for high quality GF foods, including GF beer, has increased strongly and their production has been becoming an important socio-economic issue. Despite the fact that beer is not an essential part of human nutrition, the availability of safe, healthy and tasty GF beer would appreciably improve peoples' well-being and perception of a normal social life (Hager et al., 2014). In this scenario, the global GF beer market is expected to grow at a Compound Annual Growth Rate (CAGR) greater than 40% over the period 2017–2023.

The main strategies for producing GF beer are: i) the use of naturally GF grains (i.e. rice, corn, sorghum, millet) or pseudocereals (i.e. quinoa, buckwheat, amaranth) as raw materials (Wiedemair, Ramoner, & Huck, 2019), thus obtaining beer surrogates; ii) the precipitation of proteins using tannins, silica gel or PVPP (Benìtez, Acquisgrana, Peruchena, Sosa, & Lozano, 2016; Dostalek, Hochel, Mendez, Hernando, &

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https://doi.org/10.1016/j.foodcont.2019.106987
Received 20 July 2019; Received in revised form 6 November 2019; Accepted 8 November 2019
Available online 11 November 2019
0956-7135/

Gabrovska, 2006; Hager et al., 2014; Watson, Vanderputten, Van Landschoot, & Decloedt, 2019); iii) the degradation of gluten during the brewing process by enzymatic treatments (Arendt & Zannini, 2013; Dostalek et al., 2006; Guerdrum & Bamforth, 2012; Hager et al., 2014; Watson et al., 2019). Recently, a controlled hydrocavitation-assisted brewing technique has been applied for gluten reduction in beer (Albanese, Ciriminna, Meneguzzo, & Pagliaro, 2017), as well as the use of silica gel during several beer brewing stages (Benìtez et al., 2016). Prolyl endopeptidase from *Aspergillus niger* (AN-PEP), currently used in the brewing industry for haze prevention, breaks down the proline-rich prolamin fraction of gluten. This exogenous enzyme has been successfully applied, in free form, for the production of GF beer from conventional malts (Akeroyd et al., 2016; Watson et al., 2019) without negatively impacting foam stability (Di Ghionno, Marconi, Sileoni, De Francesco, & Perretti, 2017; Guerdrum & Bamforth, 2012).

It is recognized that free enzymes usually have poor stability under process conditions (i.e., pH, temperature, and natural inhibitors in food matrix) and they cannot be recovered/reused. As reported in literature, enzyme immobilization, obtained by fixing enzyme on an appropriate carrier, is believed to provide an excellent base for improving the environmental tolerance and the operational stability of the biocatalyst in food matrix (Tang, Peng, Li, Meng, & Liu, 2018). In addition, enzyme immobilization on solid supports could allow its reuse for many cycles, also in continuous processes, avoiding protein contamination of the product (Gardossi et al., 2008).

Recently, Zhao et al. (2017) covalently immobilized AN-PEP on nonporous silica nanoparticles functionalized with amino groups, proving its efficiency in preventing chill-haze formation in beer. Despite these results, no studies have yet been carried out applying immobilized AN-PEP in a continuous bioreactor for the reduction of gluten content in beer. Among continuous bioreactors, fluidized bed reactor (FBR), is especially recommended when the substrates are viscous or contain suspended particles (Gòmez et al., 2007). In this reactor, the flow of substrate keeps the immobilized enzyme particles in a fluidized state, thus obtaining a high catalytic surface area. FBRs have widespread application in the food industry [i.e. for apple juice clarification (Diano et al., 2008), for the production of fructo-oligosaccharides and invert sugars (Lorenzoni et al., 2015), for lactose hydrolysis (Roy & Gupta, 2003), for the flavour enhancement of beverages (Gueguen, Chemardin, Pien, Arnaud, & Galzy, 1997) and for controlling malolactic fermentation in wine (Cappannella et al., 2016)].

Therefore, in this study AN-PEP has been covalently immobilized on a food-grade carrier and its environmental tolerance (temperature and pH), as well as its catalytic properties have been investigated toward a synthetic peptide substrate in synthetic beer. Afterwards, the biocatalyst was used for the first time in a continuous FBR for the production of GF conventional barley malt beer.

2. Materials and methods

2.1. Materials

Brewers Clarex® (lot No. 817786501, DSM, Delft, The Netherlands), a commercial proline-specific peptidase preparation from *Aspergillus niger* (AN-PEP), was kindly given by IMCD Italia SpA (Milan, Italy). The chromogenic para-nitroanilide (pNA) peptide Z-Gly-Pro-pNA was purchased from Bachem (Bubendorf, Switzerland) and used as synthetic substrate for protease activity. Beads as carriers for AN-PEP immobilization were produced using chitosan powder from *Aspergillus niger* (lot No. 12121611L4, KitoZyme S.A., Herstal, Belgium). Polydialdehyde starch (polymeric dialdehyde), used as a cross-linker, was purchased from Carbosynth Limited (UK). A Pilsner-type beer, with an alcohol level of 5% v/v, was kindly supplied by Free Lions Brewery (Viterbo, Italy) and the RIDASCREEN Gliadin competitive kit ELISA (Art. No. R7021), used for the quantification of gluten content in beer, was purchased from R-Biopharm (Milan, Italy). All other chemicals were of analytical grade and were from Sigma–Aldrich (Milan, Italy).

2.2. Chitosan beads preparation

Carriers for AN-PEP immobilization were prepared according to the precipitation method (Birò, Nemeth, Sisak, Feczko, & Gyenis, 2008) with some modification. Chitosan powder (15 w/v %) was dissolved in an aqueous solution of acetic acid (5 v/v%) and kept, under stirring conditions, until complete solubilisation was achieved. The solution was then added dropwise, through a peristaltic pump (Minipuls 3 Gilson, Italy), into a gently stirred coagulation liquid (2 M sodium hydroxide and 26 v/v% ethanol). The obtained beads were filtered and washed with distilled water.

2.3. Immobilization procedure

The functionalization of chitosan beads with polydialdehyde starch cross-linker was carried out as described by other authors (Benucci, Mazzocchi, Lombardelli, Cacciotti, & Esti, 2019; Chen et al., 2010). The activated beads were collected and washed with distilled water.

Afterwards, the covalent linkage of the AN-PEP was achieved by dipping the functionalized beads (1 g) in the immobilization solution (10 mL), which consisted of 0.2 M sodium phosphate buffer pH 7 containing different initial AN-PEP protein concentrations (0.1–4.6 mg_{BSAeq}/mL). After shaking at 150 rpm overnight at 20 °C, the unbound protein was removed from the carriers by extensively washing with ammonium sulphate (2 M) and synthetic beer (0.1 M sodium acetate buffer pH 4.5, containing 5% v/v ethanol).

The immobilization yield (IY, %) was indirectly determined by the protein concentration change of the immobilization solution before and after loading on chitosan carriers. The protein concentration was measured by Bradford's method (Bradford, 1976), using BSA as standard protein.

2.4. Electrophoretic separation (SDS-PAGE)

In order to demonstrate the stability of the linkage between the functionalized chitosan beads and AN-PEP, the presence of enzymatic proteins into the last washing solution was investigated by SDS-PAGE (Laemmli, 1970). It was performed on precast commercial gels of 4–15% (Bio-Rad, Richmond, California, USA), using a vertical electrophoresis apparatus (Mini-Protean Tetra cell, Bio-Rad, Richmond, California, USA); standard molecular weight (Precision Plus Protein Standards, Kaleidoscope, Bio-Rad, Richmond, California, USA) were from 10 to 250 kDa. All samples were prepared by adding 20 μL of the last washing solution to 20 μL of loading buffer (4 × Laemmli Sample Buffer), and then boiling at 90 °C for 5 min. For each sample, 20 μL was loaded in each well and the run was conducted until the dye front reached the reference line (about 1 h at 150 V). The gel was fixed and stained with colloidal Coomassie Brilliant Blue G-250, and then destained in acetic acid:methanol:water solution (9:10:81).

2.5. AN-PEP activity assay

The proteolytic activities of free and immobilized AN-PEP were evaluated in synthetic beer (0.1 M sodium acetate buffer pH 4.5, containing 5% v/v ethanol) toward the synthetic dipeptide chromogenic substrate Z-Gly-Pro-pNA [previously dissolved in 1,4-dioxane (40%, v/v in water) at 60 °C (Lopez & Edens, 2005; Zhao et al., 2017)]. The reaction product was monitored spectrophotometrically at 410 nm, measuring the change in absorbance *vs* time (2 min for free AN-PEP and 10 min for immobilized AN-PEP) by an UV–visible spectrophotometer (Shimadzu UV 2450, Milan, Italy). A blank correction was carried out using a sample without enzyme. All analysis were taken in triplicate.

When the effect of temperature and pH on the proteolytic activity of free and immobilized AN-PEP was studied, the results were expressed as

relative activity (%), taking as reference (100%) the highest activity of each enzyme under their optimal conditions.

For the kinetic characterization, the specific activity of AN-PEP was calculated in I.U. of pNA produced [$\varepsilon_{mmol} = 8.480\ mM^{-1}\ cm^{-1}$ for pNA, as reported by Hale, Greer, Trinh, and James (2005)], and it was indicated as I.U./mg of free or immobilized protein, expressed as BSAeq, (I.U./mg_{BSAeq}). The activity comparison of the free and immobilized AN-PEP was carried out at pH 4.5 at their optimum temperature and at 20 °C.

2.6. Optimum pH and temperature

The effects of temperature and pH on the proteolytic activity of free and immobilized AN-PEP towards Z-Gly-Pro-pNA spiked into synthetic beer at a concentration of 1 mM was investigated under the same assay conditions described in paragraph 2.4. AN-PEP activity assay was carried out at different temperatures, ranging from 10 °C to 80 °C, and the interval chosen for pH was between 3 and 6.

2.7. Kinetic characterization of free and immobilized AN-PEP

A kinetic study of AN-PEP free and immobilized on chitosan beads was carried out at their optimum temperatures in synthetic beer fortified with Z-Gly-Pro-pNA (0–1.5 mM) both at 20 °C and at their optimum temperature. Kinetic parameters were determined according to the Michaelis-Menten equation using a non-linear regression procedure (GraphPad Prism 5.01, GraphPad Software, Inc.).

The K_M (Michaelis-Menten constant) value reflects the enzyme substrate complex formation, whereas k_{cat} (turnover number) measures the number of substrate molecules turned over per enzyme per minute. Moreover, k_{cat} is indicative of the product release velocity, representing the maximum number of moles of substrate converted into the product per number of moles of catalyst per unit time. In addition, as K_a (affinity constant) is the ratio k_{cat}/K_M and reflects the affinity of the enzyme toward the substrate, it is indicative of both reaction steps and expresses the overall catalytic efficiency. The goodness of-fit for each data set (to their best-fit theoretical kinetic curves) was assessed as the square of the correlation coefficients (R^2).

2.8. Optimization of enzymatic treatment in the fluidized bed-reactor

The FBR consisted of a cylindrical glass column (total volume of 300 mL), equipped with an external water jacket for temperature control and connected to a thermostat (MPM INSTRUMENT TYPE M 900-TI). The use of a peristaltic pump (Pumpdrive 5206 Heidolph), and the simultaneous nitrogen (N_2) gas insufflation (1 bar flow) from the bottom of the column, allowed the suspension of the immobilized biocatalysts, as well as the recirculation of the authentic beer. Process parameters were optimized at 20 °C, varying the flow rate. The concentration of product release (C_P, given by the ratio between the ΔAbs/min and molar extinction coefficient of free pNA) was detected. Moreover, the space velocity (S_v given by the ratio between Q_v and FBR volume), the residence time (τ, corresponding to $1/S_v$) and the product-formation rate [(R_p) corresponding to C_P x Q_v] were calculated.

In order to evaluate the proteolytic activity of immobilized AN-PEP, preliminary trials were conducted in FBR at 20 °C. Authentic beer brewed with barley malt (300 mL), fortified with Z-Gly-Pro-pNA (0.12 mM), was treated with 10 g (wet weight, corresponding to 0.0036 g_{BSAeq}) of immobilized AN-PEP at different flow rates (Q_v, 112–1064 mL/min).

2.9. Gluten reduction in the fluidized bed-reactor

A Pilsner-type beer, with an initial gluten content of 65 mg/kg [(determined by a competitive ELISA based on the R5 antibody (RIDASCREEN® Gliadin competitive))], was treated in the FBR containing 10 g (wet weight) of immobilized AN-PEP, at the suitable flow rate (Q_v = 728 mL/min).

The continuous process was conducted at 20 °C for 10 h, and its efficiency in gluten reduction was evaluated collecting 1 mL of treated beer at regular time intervals (each hour) for the quantification through competitive ELISA.

2.10. Gluten quantification by competitive ELISA

A competitive ELISA based on the R5 antibody (RIDASCREEN® Gliadin competitive) was used for the quantification of gluten content in beer before, during and after the treatment in FBR.

A beer sample (1 mL) was mixed with 9 mL of the extraction solution [60% (v/v) ethanol in water containing 10% (m/v) of fish gelatin] and analyzed according to the manufacturer's instructions. The recommended dilution (1:500) was used for each sample, meaning that only gluten contents between 10 and 270 mg/kg could be determined. Absorbances at 450 nm were measured using an Asys Expert 96 microplate reader (Biochrom, Cambridge, UK), and prolamin concentrations were obtained by using RIDA®SOFT Win software (R-Biopharm). Prolamin concentrations were converted into gluten content by multiplication by factor 2, as provided by Codex Alimentarius Commission (Commission, C. A., 2008). All samples were analyzed in triplicate.

2.11. Statistical analysis

The data, obtained from the average of three replicate analyses, were analysed by a one-way completely randomized Analysis of Variance (ANOVA). When significance was revealed, a Tukey's (HSD) post-hoc test ($p < 0.05$) was performed using EXCEL® Add-in macro DSAASTAT program for multiple comparisons of samples (Onofri, 2006).

3. Results and discussion

3.1. AN-PEP immobilization

A first experiment was carried out in order to determine the effect of the protein concentration in the immobilization solution (ranging from 0.1 to 4.6 mg_{BSAeq}/mL) on the protein loaded, as well as on the specific activity of immobilized AN-PEP.

Data in Fig. 1 show that protein loading significantly increased when higher initial protein concentrations were used, until reaching the maximum value (about 7 $mg_{BSAeq}/g_{chitosan}$ using an immobilization solution at 3 mg_{BSAeq}/mL). The further increase of the protein concentration in the immobilization solution did not significantly affect the amount of immobilized protein.

The specific activity of AN-PEP increased as the concentration of the immobilized solution increased, reaching a maximum (0.016 I.U./mg_{BSAeq}) value when the procedure was carried out using an initial protein concentration of 0.3 mg_{BSAeq}/mL. Although a further increase in initial protein concentrations resulted in a greater amount of protein loaded, AN-PEP specific activity decreased markedly. As reported in literature, the amount of protein immobilized on carriers has obvious influence on the performance of the immobilized enzyme (Zhang, Yuwen, & Peng, 2013). Excessive enzyme loading causes protein-protein interaction and inhibits the flexible stretching of enzyme conformation, which results in the steric hindrance responsible for the enzyme inactivation (Tischer & Wedekind, 1999; Zhang et al., 2013).

The SDS-PAGE (on the last washing solution after the immobilization) showed that regardless of the initial protein concentration in the immobilization solution, bands of molecular weight similar to those of free AN-PEP (used as reference standard) were not observed (data not shown). This can be an indication of the stability of the linkage between the functionalized carriers, based on chitosan from *A. niger*, and AN-PEP enzyme.

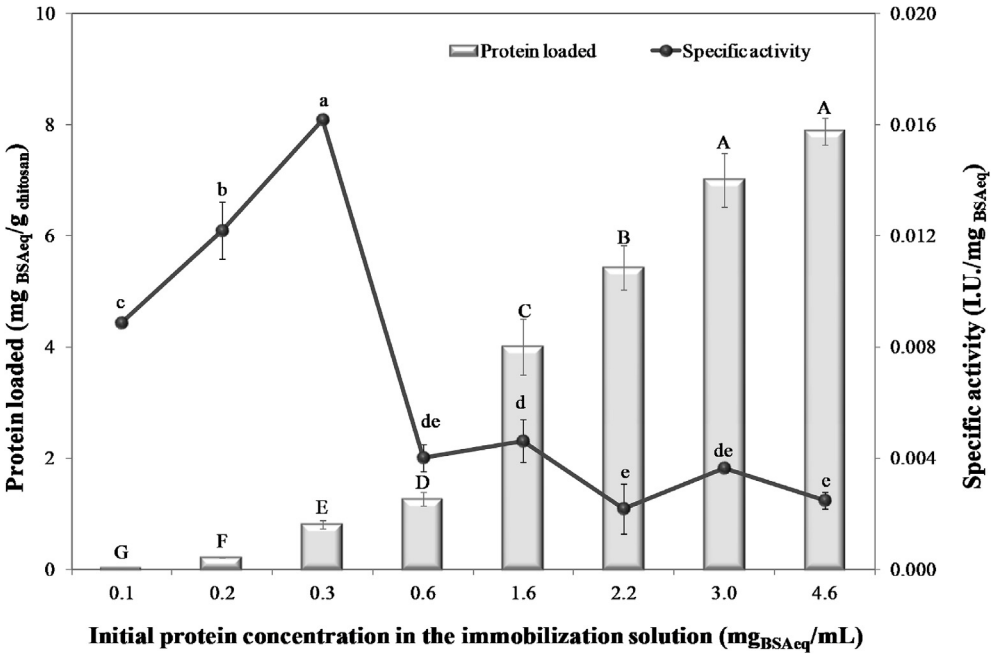

Fig. 1. Protein loaded ($mg_{BSAeq}/g_{chitosan}$) and specific activity ($I.U./mg_{BSAeq}$) of immobilized AN-PEP obtained by varying the initial protein concentration in the immobilization solution (mg_{BSAeq}/mL). The proteolytic activity was assayed in synthetic beer (0.1 M sodium acetate buffer pH 4.5, containing 5% v/v ethanol) toward the synthetic dipeptide chromogenic substrate, Z-Gly-Pro-pNA (0.5 mM). Different capital letters indicate significant differences in protein loaded, whereas small letters indicate significant differences in specific activity (Tukey's test, $p < 0.05$).

3.2. Temperature and pH optima of free and immobilized AN-PEP

The influence of temperature and pH on the activity of free and immobilized AN-PEP (Fig. 2), investigated in synthetic beer, proved that all the curves exhibited the typical bell-shape trend (Bisswanger, 2014). It was found that the optimum temperature for the free AN-PEP was 60 °C, corresponding to a specific activity of 0.163 ± 0.006 I.U./mg_{BSAeq} (Fig. 2a). After immobilizing the protease on chitosan beads, the optimum temperature was broadened to a range of 50–60 °C, and the highest relative activity was at 50 °C (corresponding to a specific activity of 0.0085 ± 0.0002 I.U./mg_{BSAeq}, Fig. 2a). AN-PEP had the same optimum temperature (50 °C) when it was immobilized on non-porous silica nanoparticles (Zhao et al., 2017). The effect of different pH values was investigated both at 20 °C (temperature conventionally applied in brewing during the post-fermentation step) and at the optimum temperature for free (60 °C) and immobilized enzyme (50 °C). Regardless of the temperature, immobilized AN-PEP maintained a suitable proteolytic activity in the typical range of beer pH, showing the optimum pH at 4.2–4.5 (with a specific activity of about 0.017 ± 0.002 I.U./mg_{BSAeq} at 20 °C, and a specific activity of about 0.021 ± 0.001 I.U./mg_{BSAeq} at 60 °C) (Fig. 2b and c).

3.3. Kinetic characterization of free and immobilized AN-PEP

The kinetic study, carried out in synthetic beer both at 20 °C and at the optimum temperature (60 °C for free and 50 °C for immobilized AN-PEP), proved that both biocatalysts followed the hyperbolic behavior of the Michaelis-Menten equation (Fig. 1S). Regardless of the temperature, the immobilization considerably reduced the catalytic properties of the biocatalyst, with a significant decrease of all the kinetic parameters (Table 1). This reduction, after covalent immobilization, is a common phenomenon and it could be attributable to steric hindrance, diffusion issues as well as to the low amount of enzyme immobilized on chitosan supports (Jiang, Long, Huang, Xiao, & Zhou, 2005).

No significant differences were revealed comparing the kinetic parameters of immobilized AN-PEP applied at 20 °C or at its optimum value (50 °C), suggesting that immobilization increases the thermal stability of the protease, as already ascertained for other enzymes (Datta, Christena, & Rajaram, 2013). These results prove that the catalytic properties of immobilized AN-PEP were not affected by the operating temperature. Otherwise, the catalytic properties of free enzyme were significantly influenced by the operating temperature, with the highest catalytic efficiency observed when free AN-PEP was used at its optimum temperature (60 °C). At 20 °C all catalytic properties of free enzyme were significantly better than those of immobilized enzyme, with the only exception of K_M.

3.4. Treatment optimization in fluidized-bed reactor

The flow rate (Q_v) changing in FBR resulted in a corresponding variation of the other process parameters, such as S_v and τ (Table 2). As expected, a positive linear relationship between Q_v and S_v was revealed, whereas an inverse relationship between Q_v and τ was observed, thus indicating that at higher flow rates the contact-time between the immobilized enzyme and the substrate deeply diminished. Moreover, data in Table 2 show that the relationship between Q_v and R_p was represented by a bell shape curve (Fig. 3), as described by other authors (Laudani, Habulin, Knez, Della Porta, & Reverchon, 2007; Saponjić et al., 2010).

Immobilized AN-PEP showed a satisfactory proteolytic activity in authentic beer fortified with the synthetic peptide substrate. The highest product-formation rate (R_p = 0.073 μM/mg_{BSAeq}) was revealed at a Q_v of 728 mL/min (corresponding to a τ of 0.32 min) (Fig. 3). The further increase of Q_v, at values higher than 728 mL/min, resulted in an evident reduction of the product-formation rate, probably due to the greater turbulence at high flow rates, as well as to the reduction of contact-time between the immobilized protease and the substrate, leading to the decrease of the R_p (Saponjić et al., 2010; Zhou, Chen, & Yan, 2014). Thus, the suitable Q_v to achieve the gluten reduction in authentic beer through treatment in FBR was 728 mL/min.

3.5. Effect of continuous treatment in fluidized bed reactor on beer gluten content

As described in literature, a natural gluten reduction takes place throughout the brewing process (Di Ghionno et al., 2017; Watson et al., 2018). First of all, a significant decrease usually occurs during the malting and mashing, when activated barley endogenous proteinases hydrolyse gluten partially to peptides or completely to amino acids (Colgrave, Goswami, Howitt, & Tanner, 2011; Hager et al., 2014;

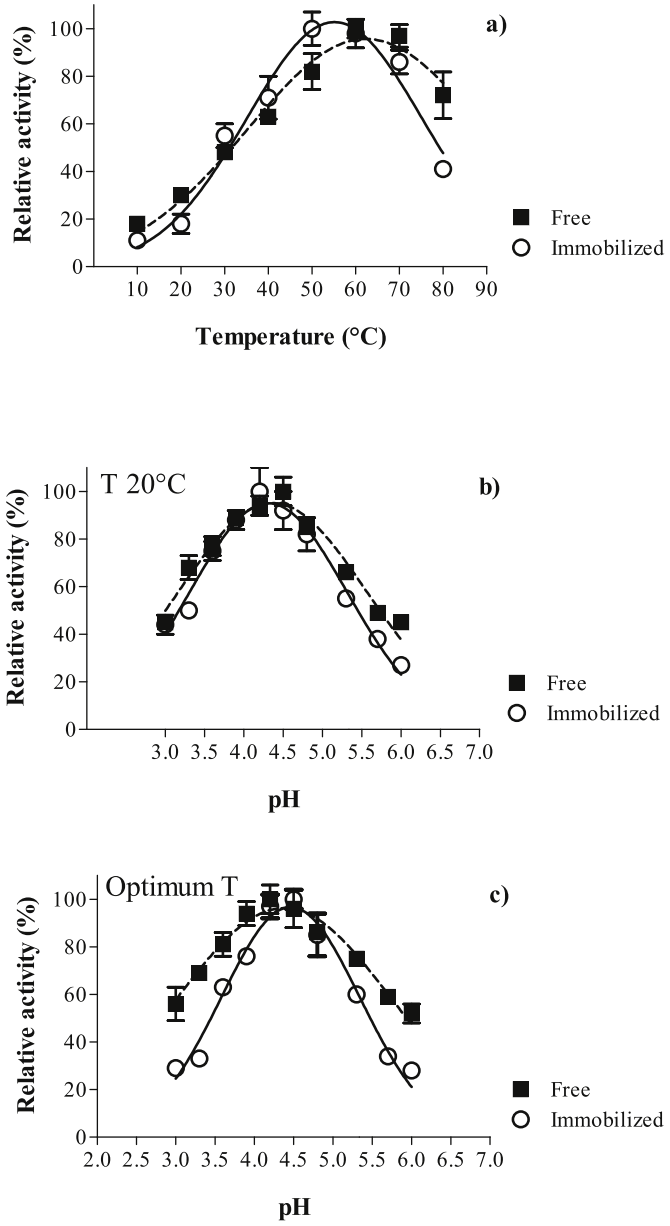

Fig. 2. Effect of temperature (**a**) and pH [at 20 °C (**b**) or at the optimum T (**c**)], on the proteolytic activity of free (■) and immobilized AN-PEP (○). The proteolytic activity was evaluated in synthetic beer (0.1 M sodium acetate buffer, containing 5% v/v ethanol) toward the synthetic dipeptide chromogenic substrate, Z-Gly-Pro-pNA (1 mM).

Tanner, Colgrave, & Howitt, 2014; Van Landschoot, 2011). Moreover, throughout the lautering step the gluten reduction is mainly due to protein precipitation (Steiner, Gastl, & Becker, 2011). A further decrease takes place during the primary fermentation of wort, when much of this proteinaceous material in wort is removed with the spent grains and hot-break (Kunze, 2004) or is taken up by yeast during fermentation for nitrogen metabolism (Lekkas, Hill, Taidi, Hodgson, & Stewart, 2009; Mo, Zhao, Lei, & Zhao, 2013; Van Landschoot, 2011). Thereafter, reduction in gluten content throughout the maturation phase is likely due to the low temperatures which promote the further protein-polyphenol complexes sedimentation.

Table 2
Process parameters of fluidized-bed reactor containing immobilized AN-PEP (10 g), evaluated in authentic beer toward the synthetic dipeptide chromogenic substrate [Z-Gly-Pro-pNA (0.12 mM)], at 20 °C and at different flow rates (Q_v, 112–1064 mL/min).

Q_v (mL/min)	S_v (1/min)	τ (min)	R_p (μM/mg_{BSAeq})
112	0.48	2.08	0.011
224	0.97	1.04	0.013
392	1.69	0.59	0.014
560	2.41	0.41	0.033
728	3.14	0.32	0.073
894	3.85	0.26	0.020
1064	4.59	0.22	0.018

Q_v [Flow rate (mL/min)]; S_v [Space velocity (1/min)]; τ [Residence time (min)]; R_P [product-formation rate (μM/mg_{BSAeq})].

For this reason the beer treatment in FBR, containing immobilized AN-PEP, was conducted on a commercial Pilsner-type beer following the maturation step. The initial gluten content (65 mg/kg) slowly reduced, until reaching the level of 19 mg/kg after 9 h and 15 mg/kg after 10 h of treatment (Fig. 4). Immobilized AN-PEP, applied in the continuous FBR, was able to degrade the gluten content at a concentration lower than 20 mg/kg, which corresponds to the amount established by the Codex Alimentarius Standard and the EU-regulation 828/2014 for GF food (Shan, 2002).

Despite these encouraging results, the presence of immunogenic gluten fragments in the treated beer (with a final gluten content of 15 mg/kg) could not be excluded. Fiedler et al. (2019) recently proved that gluten peptides, containing potential immunopathogenic sequences, have been identified in beers treated by using AN-PEP in free form, followed by filtration. In this context, the use of mass spectrometry techniques to detect gluten peptide in gluten-reduced beers, as suggested by other authors (Colgrave et al., 2014, 2017; Tanner, Colgrave, Blundell, Goswami, & Howitt, 2013), could supply valuable information about the nature and the relative concentration of gluten and gluten-derived peptides.

4. Conclusions

The production of high quality gluten-reduced beer remains a challenging problem, which is worthy of serious exploration in order to find better useful approaches to be applied in brewing.

In this study, we have demonstrated that the enzyme prolyl endopeptidase from *Aspergillus niger* (AN-PEP), currently used in the brewing industry, could be applied for the industrial production of GF beer also in immobilized form. The continuous treatment of beer in a fluidized bed reactor (FBR), containing the novel and food-grade immobilized biocatalyst, allowed the reduction of gluten content in the

Table 1
Kinetic parameters of free and immobilized AN-PEP, in synthetic beer (0.1 M sodium acetate buffer pH 4.5, containing 5% v/v ethanol), toward the synthetic dipeptide chromogenic substrate, Z-Gly-Pro-pNA (0–1.5 mM), at 20 °C and at the optimum T (60 °C for free and 50 °C for immobilized AN-PEP).

AN-PEP	V_{max} (I.U./mg_{BSAeq})	K_M (mM)	k_{cat} (1/min)	K_a (1/min* × mM)	R^2
Free $_{20\,°C}$	0.0937 ± 0.0025 [b]	0.210 ± 0.019[b]	14059 ± 20 [b]	66918 ± 6026 [b]	0.98
Immobilized $_{20\,°C}$	0.0058 ± 0.0003 [c]	0.047 ± 0.011 [c]	0.58 ± 0.03 [c]	12 ± 3 [c]	0.92
Free $_{T\ optimum\ (60\,°C)}$	0.5474 ± 0.0324 [a]	0.643 ± 0.082 [a]	82110 ± 40 [a]	127738 ± 16602 [a]	0.98
Immobilized $_{T\ optimum\ (50°C)}$	0.0063 ± 0.0002 [c]	0.038 ± 0.006 [c]	0.63 ± 0.02 [c]	17 ± 3 [c]	0.96

*Values with different small letters differ significantly (Tukey's test, $p < 0.05$).

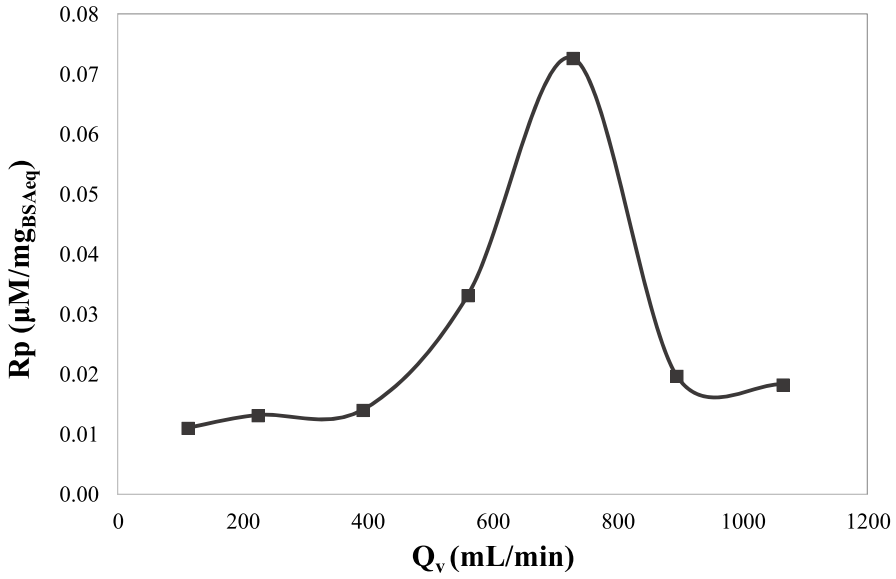

Fig. 3. Relationship between flow rate (Q_v) and product-formation rate (Rp) in fluidized-bed reactor (FBR), containing immobilized AN-PEP (10 g), evaluated in authentic beer toward the synthetic dipeptide chromogenic substrate [Z-Gly-Pro-pNA (0.12 mM)], at 20 °C and at different flow rates (Q_v, 112–1064 mL/min).

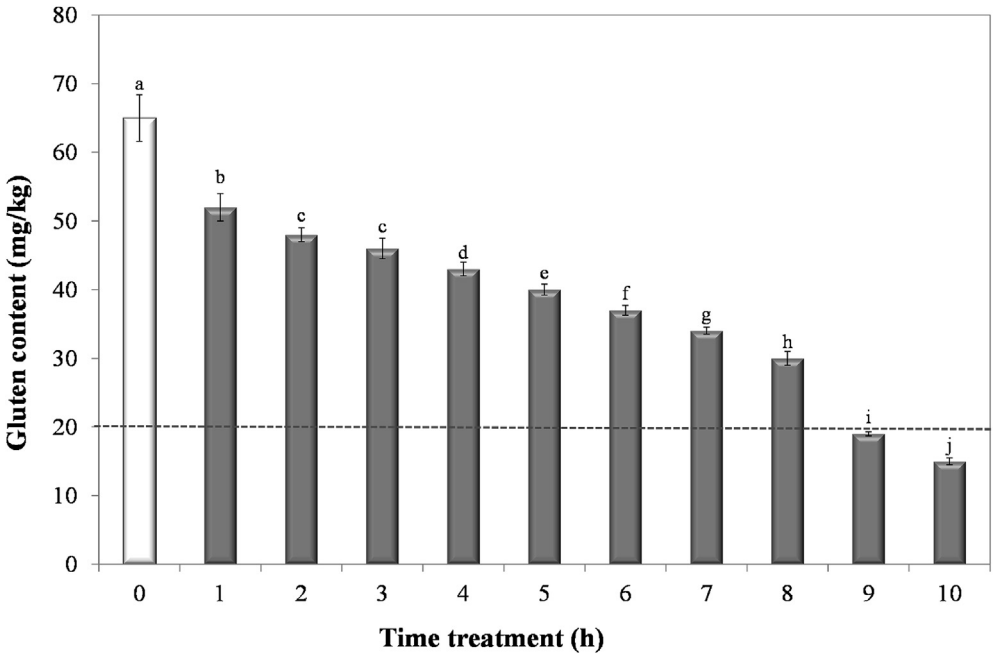

Fig. 4. Changes in the gluten content (mg/kg) of Pilsner-type beer during the treatment in FBR, containing 10 g of immobilized AN-PEP, at 20 °C and flow rate of 728 mL/min.
Different letters indicate significant differences in gluten amount (Tukey's test, $p < 0.05$).

commercial beer from barley malt, reaching the level under the minimum (< 20 mg/kg) requested for GF food. From an industrial point of view, this preliminary study opens a potential new biocatalytic approach for the continuous production of GF beer.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgement

This work was supported by BioEnBi project "Biotecnologie enzimatiche innovative per processi di chiarifica sostenibili nel settore birrario", funded by Lazio Region (Italy) in the context of Progetti Gruppi di Ricerca, LazioInnova 2018–2020.

We are particularly grateful to Free Lions Brewery (Viterbo, Italy) which kindly supplied the Pilsner-type beer.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2019.106987.

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