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ORIGINAL PAPER

Antithrombotic Activity of Brewers' Spent Grain Peptides and their Effects on Blood Coagulation Pathways

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Abstract

Antithrombotic activity of brewers' spent grain peptides before and after simulated gastrointestinal digestion and their effects on blood coagulation pathways were evaluated. Two hydrolysates were produced using sequential enzymatic systems: alkaline protease + Flavourzyme (AF) and neutral protease + Flavourzyme (PF). Simulation of gastrointestinal digestion of AF and PF hydrolysates was made using porcine pepsin and pancreatin enzymes, obtaining the corresponding digested samples: AFD and PFD, respectively. Peptides were fractionated by ultrafiltration using a 1 kDa cut-off membrane. Hydrolysates had peptides with medium and low molecular weights (2100 and 500 Da, respectively), and Glu, Asp, Leu, Ala, and Phe were the most abundant amino acids. Gastrointestinal digested hydrolysates presented high proportion of small peptides (~500 Da), and higher amount of Val, Tyr, and Phe than hydrolysates. Mass spectrum (HDMS Q-TOF) of AFD-ultrafiltered fraction <1 kDa exhibited peptides from 500 to 1000 Da, which are not present in AF. PFD showed the generation of new peptides from 430 to 1070 Da. All samples showed thrombin inhibitory activity. However, no effect was observed on prothrombin time. Peptides <1 kDa from hydrolysates and digested samples delayed thrombin and thromboplastin time respect to the control (~63%). Also the samples showed thrombin inhibitory activity at common pathway level. Thus, brewers' spent grain peptides exerted their antithrombotic activity by inhibiting the intrinsic and common pathways of blood coagulation. This is the first report to demonstrate that brewers' spent grain peptides are able to delay clotting time after simulated gastrointestinal digestion.

Keywords Multi-enzyme bioactive peptide extraction · Antithrombotic peptides · ACE I and α-amylase inhibition mechanism · *In vitro* gastrointestinal digestion · Brewers' spent grain

Abbreviations

ACE I	Angiotensin-converting enzyme
AF	Hydrolysate obtained with alkaline protease during 2 h + Flavourzyme 2 h
AFD	Gastrointestinal digested sample from AF
BSG	Brewers' spent grain
ESM	Electronic supplementary material
PF	Hydrolysate obtained with neutral protease during 2 h + Flavourzyme 2 h
PFD	Gastrointestinal digested sample from PF

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Introduction

Brewer's spent grain (BSG) is the main by-product generated during the beer production process. The spent grains are separated from beer wort by filtration after the mashing phase. They correspond to the insoluble fraction of the wort, essentially composed by a lignocellulosic material containing protein (~30% on a dry weight basis), lignin (~28%), hemicelluloses (~25%), and cellulose (~17%) [1, 2]. Currently, the main application of BSG is limited to animal feed. However, due to

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the fact that this byproduct is available at low or no cost through the year and it presents a nutritional value, BSG is a promising raw material to be exploited for human nutrition and biotechnological processes [3]. In addition, the importance of BSG as an ingredient and potential source of health-promoting bioactive components is beginning to be recognized [4].

Protein hydrolysates have numerous uses in human nutrition ranging from protein supplementation of geriatric and sports nutrition products, energy drinks, and weight-loss diets to clinical applications including treatment of Crohn's disease, liver disease, and ulcerative colitis [2, 5]. In this regard, different BSG protein hydrolysates have been shown to exert bio-functional properties such as: antioxidant, anti-microbial, anti-inflammatory effects, α-glucosidase, α-amilase, angiotensin converting enzyme I (ACE I), and dipeptidyl peptidase-IV inhibitory properties [2, 3, 6–11]. However, antithrombotic activity of BSG peptides is an unexplored field yet to be informed. Moreover, as far as we know, there is no research about the effect of BSG peptides on blood coagulation pathways and even less about stability after *in vitro* gastrointestinal digestion using porcine enzymes. Therefore, the aim of this work was to evaluate the antithrombotic activity of brewers' spent grain peptides before and after simulated gastrointestinal digestion and their effects on blood coagulation pathways. Furthermore, α-glucosidase, α-amylase, and angiotensin-converting enzyme inhibitory activities were evaluated.

Materials and Methods

This section is in the Online Resource (ESM 1).

Results and Discussion

Characterization of Hydrolysates and Gastrointestinal Digested

The degree of hydrolysis (DH) of AF and PF were 17.1 ± 0.5 and 11.4 ± 0.7%, respectively. Similar DH values were found by Connolly et al. [12], who studied the techno-functional properties of brewers' spent grain hydrolysates obtained with different enzymatic systems (Alcalase, Corolase PP, Flavourzyme, and Promod 144MG). The higher protein content and DH of AF system can be attributed to the alkaline protease enzyme (A) and hydrolysis pH. Niemi et al. [13] found Alcalase (an alkaline protease) proved to be the most efficient protease solubilizing 35% of proteins from BSG at pH 9.5 within 4 h, whereas with neutral and acid proteases the degree of solubilization was only 14%. In agreement with this, AF system solubilizing 38.3% of proteins from BSG at pH 9.5 within 4 h, whereas with PF system the degree of solubilization was only 14.5%.

Figure 1 shows the FPLC gel filtration profile of BSG hydrolysates and gastrointestinal digested hydrolysates. AF and PF presented four main peaks of >7000 Da, 2100, ~500, and, 120 Da, respectively. The components higher than 7000 Da presented an elution volume higher than that corresponding to exclusion volume. The peak of 2000 Da corresponds to intermediate MW species, while 500 and 120 Da peaks correspond to peptides of low MW and free amino acids, respectively (Fig. 1a and b). Niemi et al. [13] reported that enzymatically solubilized proteins had a molecular weight (MW) lower than 15 kDa. McCarthy et al. [2] found that GP-HPLC profile of hydrolysates showed that up to 80% of the peptides were < 5 kDa when compared to the unhydrolyzed BSG protein rich isolate. AF profile showed a shoulder of 5000 Da which can be attributed to alkaline pH used in first step of AF hydrolysis, since alkaline conditions promote the solubilization of proteins and oligopeptides [13]. AFD and PFD profiles show effective degradation of intermediate MW peptides (2100 Da) by pepsin and pancreatin enzyme. This is evinced as a shoulder and a peak of 1900 Da or 1800 Da and 1400 or 1200 Da, respectively (Fig. 1c and d). The proportion of low MW peptides (~500 Da) increased after *in vitro* gastrointestinal digestion respect to AF and PF hydrolysates (Fig. 2a). These results agree with the higher DH values of AFD and PFD (25.4 ± 0.8 and 22.1 ± 1.1%, respectively).

Table 1 shows amino acid profile of BSG, its hydrolysates, and gastrointestinal digested hydrolysates. The most abundant amino acids in BSG, AF, and PF were Glu, Asp, Leu, Ala, and Phe. Hordeins account for 60% of the total protein in BSG and are recognized for their high Glu content. Moreover, glutelins (the barley cell wall structural proteins) comprise the bulk of the remaining protein fraction and contain high levels of Ala and Asp residues [6]. However, the most abundant amino acids for AFD and PFD were Val, Ala, Tyr, Glu, Asp, and Phe. In this regard, the ratio of Val, Tyr, and Phe respect to the contents of the respective hydrolyzate were 1.5, 1.6 and 1.5, respectively. Moreover, Pro, Leu, Asp, and Glu content of AFD and PFD were lower than BSG, AF, and PF.

Antithrombotic Activity of Hydrolysates and Gastrointestinal Digested Hydrolysates

Thrombin time (TT) evaluates the conversion of fibrinogen in fibrin through the action of thrombin, thereby the presence of substances interfering with the action of the enzyme over fibrinogen or blocking polymerization of fibrin monomers lead to its prolongation [14]. The results obtained show that all samples produced a delay in TT respect to control at 2 g/L protein (Fig. 2b). Gastrointestinal digestion increased in 98

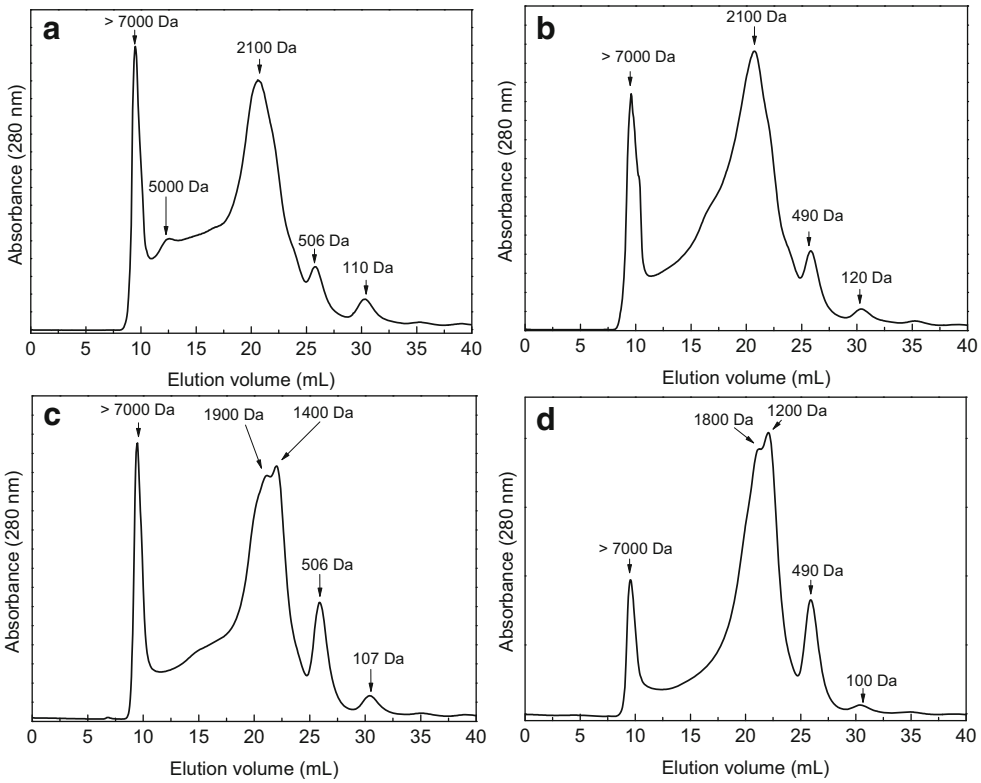

Fig. 1 FPLC gel filtration profiles of hydrolysates (AF and PF; **a** and **b** respectively) and gastrointestinal digested hydrolysates (AFD and PFD; **c** and **d** respectively). Gel filtration chromatography was carried out with a Superdex peptide column. Injection volume was 100 μL (2.8 mg protein/mL). Elution was carried out using 50 mmol/L Potasium Phosphate Buffer pH 7.0 plus 150 mmol/L NaCl at 1 mL/min. The profile shown is representative of several that were carried out

and 90% TT in AFD and PFD, respectively, increases being significantly higher than those obtained with AF and PF (42 and 34%, respectively). This effect was also observed at 1 g/L protein, indicating an independent response of both protein concentrations (results not shown). Rojas-Ronquillo et al. [15] studied the effect of gastric enzymes, pepsin and trypsin, on antithrombotic and angiotensin-converting enzyme inhibitory properties of peptides released from bovine casein by *Lactobacillus casei* Shirota. They found that four of seven peptides evaluated maintained their thrombin inhibitory activity after gastrointestinal digestion. Moreover, Jung and Kim [16] determined anticoagulant activity from marine bivalve's hydrolysates and found that the tetra-peptide Met-Glu-Ala-Pro prolonged TT.

As shown in Fig. 2c, the samples studied did not induce changes in clotting times of prothrombin time (PT) compared to control plasma at 2 g/L protein. These results do not controvert with TT ones, as the PT evaluates the extrinsic pathway of coagulation. Sabbione et al. [16] reported that amaranth proteins hydrolysates delayed the TT but had no effect on the PT at 2.22 g/L protein.

All samples produced a delay in activated partial thromboplastin time (APTT) test respect to control at 2 g/L protein (Fig. 2d). Both, AFD and PDF exhibited higher activity than AF and PF, respectively ($P > 0.05$). These results indicate that gastrointestinal digestion with pepsin and pancreatin promoted the generation of new peptides able to delay the activated partial thromboplastin time. This effect was also observed at 1 g/L protein (results not shown). Kong et al. [17] isolated three novels antithrombotic peptides from Centipedes (*Scolopendra subspinipes mutilans* L. Koch) hydrolysates. The primary structures of these peptides were Phe-Ser-Ala-Pro-Ala-Val-Tyr (753 Da), Ile-Arg-Asp-Leu (515 Da), and Asp-Leu-Asp-His-Tyr-Ser-Phe (985 Da). All isolated peptides potently prolonged the APTT. Taking into account the sizes of the isolated peptides mentioned above from different protein sources, it could be assumed that the higher delay in TT and APTT of AFD and PFD is due to low MW species lower than 1000 Da generated by pepsin and pancreatin enzymes during gastrointestinal digestion. This was corroborated by evaluating the ultrafiltered peptide fractions. In this regard, fractions with MW <1 kDa had higher TT and APTT than those with >1 kDa peptides (Fig. 2e and f, respectively). Moreover, UPLC–MS of ultrafiltered peptide fractions <1 kDa from AFD and PFD showed new peptides generated by gastrointestinal digestion (ESMs 2 and 3). Mass spectrum of AFD-ultrafiltered fraction <1 kDa exhibited peptides of molecular mass from 500 to 1000 Da, which are not present in AF (ESM 2A and B). On the other hand, PFD showed the generation of new peptides of molecular mass from 430 to 1070 Da (ESM 3A and B). These results would confirm the hypothesis that low molecular mass peptides generated after gastrointestinal digestion (400–1000 Da) are responsible for increasing the antithrombotic activity in AFD and PFD. In this sense, the information gathered in bibliography shows that peptides with antithrombotic activity tend to be short [18].

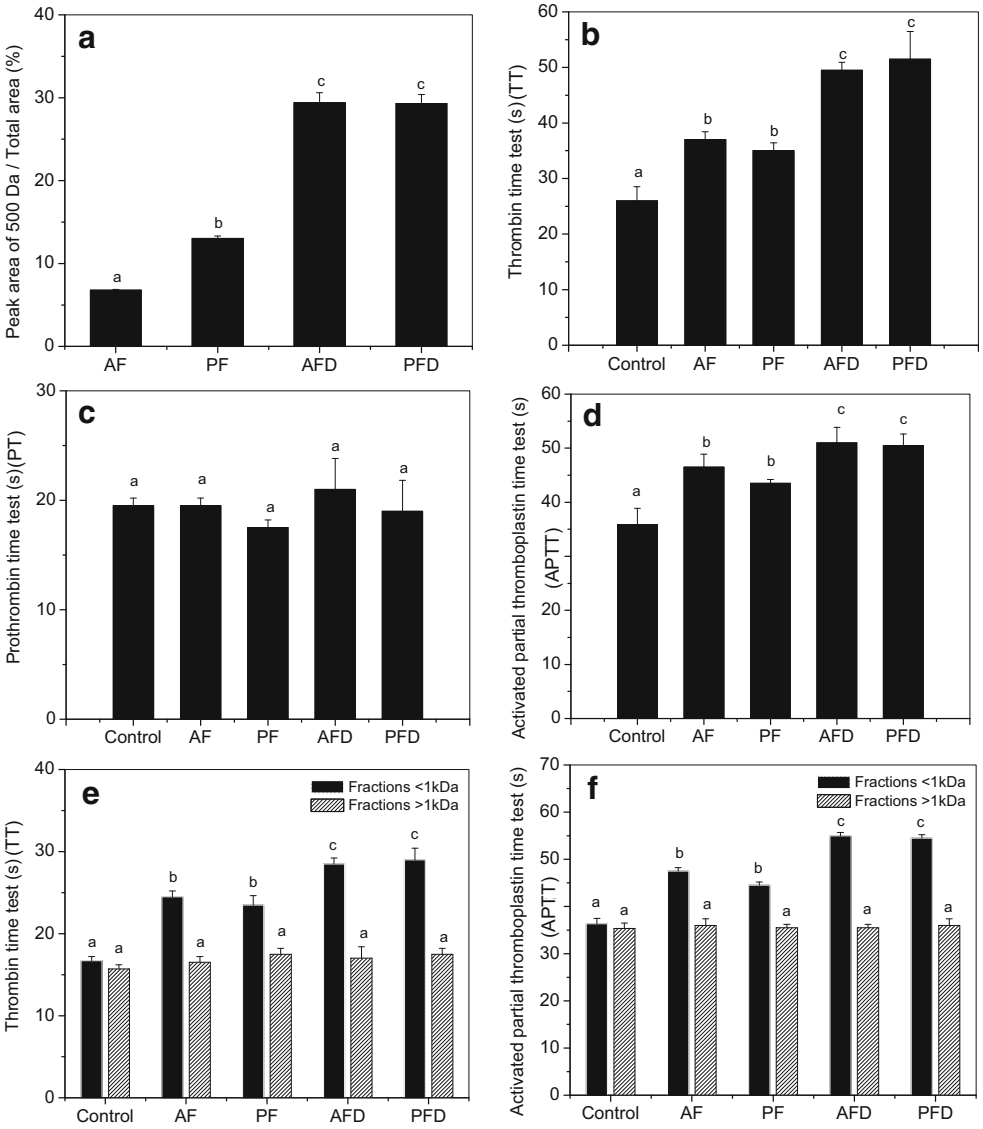

Fig. 2 Ratio between peak area of 500 Da and total area (**a**), thrombin time test-TT (**b**), prothrombin time test-PT (**c**), and activated partial thromboplastin time test-APTT (**d**) of hydrolysates and digested hydrolysates (2 g/L protein). Thrombin time test-TT (**e**) and activated partial thromboplastin time test-APTT (**f**) of ultrafiltered fractions (2 g/L protein). Control was performed with saline at the same amount as the sample. Different letters mean significant differences between samples ($P < 0.05$)

Microplate assay emulates the common way (the last stage of blood coagulation), where thrombin (FIIa) hydrolyses fibrinogen (FI) generating fibrin monomers (FIa) that subsequently polymerize [14]. In this regard, all samples inhibited the action of thrombin preventing the proteolysis of the fibrinogen. All samples showed thrombin inhibitory activity. The IC_{50} values for AF, PF, AFD, and PFD were 0.909 ± 0.03, 1.012 ± 0.040, 0.635 ± 0.01, and 0.604 ± 0.02 g/L protein, respectively. As observed, IC_{50} values of AFD and PFD were significantly lower than those found for AF and PF. Thus, the *in vitro* gastrointestinal digestion increased antithrombotic activity. The inhibitory action of hydrolysates and digested samples may be due to the union to one or both of the active sites of thrombin, preventing the proteolysis of the fibrinogen. Also, Sabbione et al. [18] reported the inhibition of thrombin for amaranth hydrolysate obtained by activation of an endogenous protease.

Previous studies indicated that the antithrombotic activity of peptides is usually associated with the presence of Ala and hydrophobic amino acid residues such as Phe and Tyr [16, 17, 19]. As mentioned above, the most abundant amino acids in AFD and PFD were Val, Tyr, and Phe, and to a lesser extent Ala. Thus, the higher antithrombotic activity of AFD and PFD could be due to the presence of these amino acids in the sequence of low MW peptides.

Regarding α-glucosidase inhibition, all samples showed inhibitory activity. The IC_{50} values of AFD and PFD were significantly lower than those found for AF and PF (ESM 4A). Thus, *in vitro* gastrointestinal digestion increased α-glucosidase inhibition. Connolly et al. [6] found tryptic digests of BSG-protein isolate resulted in the greatest α-glucosidase inhibition, attributing this effect to protease specificity which produces peptides with an Arg residue at the C-terminal. In our work we used pancreatin from porcine

Table 1 Amino acid profile and protein content of brewers' spent grain (BSG), hydrolysates (AF and PF), and gastrointestinal digested hydrolysates (AFD and PFD)

	BSG	AF	PF	AFD	PFD
Total amino acids (g 100 g^{-1} protein)					
Asp + Glu	12.2 ± 1.0^{b}	11.9 ± 1.4^{b}	12.1 ± 1.1^{b}	8.9 ± 0.8^{a}	9.1 ± 0.9^{a}
Ser	6.5 ± 0.1	6.2 ± 0.5	6.3 ± 0.9	6.2 ± 0.6	6.4 ± 0.4
His	2.9 ± 0.1	3.0 ± 0.2	2.8 ± 0.1	2.6 ± 0.3	2.7 ± 0.2
Gly	8.0 ± 0.3	7.7 ± 0.3	7.8 ± 0.3	8.1 ± 0.1	7.8 ± 0.2
Thr	5.1 ± 0.0	5.0 ± 0.1	4.7 ± 0.3	4.8 ± 0.1	5.2 ± 0.4
Arg	7.0 ± 0.0	7.3 ± 0.2	6.9 ± 0.2	7.3 ± 0.1	7.0 ± 0.0
Ala	9.8 ± 0.4	9.5 ± 0.8	9.9 ± 0.1	9.7 ± 0.4	9.8 ± 0.4
Pro	7.9 ± 0.6^{b}	7.8 ± 0.7^{b}	7.7 ± 0.7^{b}	5.6 ± 0.5^{a}	5.5 ± 0.2^{a}
Tyr	5.1 ± 0.2	5.2 ± 0.3	5.1 ± 0.2	8.2 ± 0.1	8.3 ± 0.3
Val	5.6 ± 0.1^{a}	5.5 ± 0.3^{a}	5.7 ± 0.1^{a}	8.5 ± 0.2^{b}	8.6 ± 0.1^{b}
Met	1.4 ± 0.0	1.3 ± 0.0	1.4 ± 0.1	1.3 ± 0.0	1.3 ± 0.1
Cys	2.7 ± 0.1	2.8 ± 0.3	2.6 ± 0.1	2.8 ± 0.2	2.9 ± 0.3
Ile	5.5 ± 0.1	5.8 ± 0.2	5.3 ± 0.4	5.6 ± 0.1	5.8 ± 0.4
Trp	0.5 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.4 ± 0.1	0.5 ± 0.0
Leu	10.9 ± 0.4^{b}	10.8 ± 0.3^{b}	11.0 ± 0.3^{b}	7.5 ± 0.2^{a}	7.6 ± 0.4^{a}
Phe	10.7 ± 0.6^{a}	10.7 ± 0.5^{a}	10.9 ± 0.3^{a}	16.3 ± 1.2^{b}	16.2 ± 1.0^{b}
Lys	8.7 ± 1.1	8.9 ± 0.5	8.7 ± 0.8	8.5 ± 1.0	8.7 ± 0.8
Protein (g 100 g^{-1} dw)	21.4 ± 0.1^{a}	48.3 ± 1.0^{d}	45.0 ± 1.0^{c}	33.2 ± 0.3^{b}	32.6 ± 1.0^{b}

Total amino acid and protein content expressed as mean ± SD ($n = 3$); dw (dry weight). Different letters in a row mean significant differences between samples ($P < 0.05$)

pancreas, which contains different enzymatic components such as trypsin, amylase, lipase, ribonuclease, and protease. Thus, the positive effect of *in vitro* gastrointestinal digestion on α-glucosidase inhibition could be associated to new peptides with low MW generated by pancreatin from AF and PF hydrolysates (ESM 2 and 3).

Surprisingly, neither of the two hydrolysates inhibited α-amylase activity (ESM 4B). The poor α-amylase inhibitory activity of BSG hydrolysates was also observed by Connolly et al. [6]. In that work, the authors found no significant difference in α-amylase inhibition between BSG and its hydrolysates. However, in our work, the digested hydrolysates exhibited inhibitory activity. These results suggest that pepsin and pancreatin digestion promoted the generation of new more active peptides with MW <1 kDa (ESMs 3 and 4). In this regard, Connolly et al. [7] reported that gastrointestinal digestion of Alcalase hydrolysate obtained from BSG resulted in a significant increase in DPP-IV inhibitory activity, another enzyme involved in glucose metabolism [20]. The study of inhibition mechanism using Lineweaver-Burk plots showed that peptide fractions from AFD and PFD with MW <1 kDa were uncompetitive inhibitors (ESM 4C).

The IC_{50} values for ACE I inhibition of AF, PF, AFD, and PFD were 1.4 ± 0.1, 2.3 ± 0.1, 4.4 ± 0.1, and 5.2 ± 0.2 g/L of protein, respectively. The increase of IC_{50} values of AFD and PFD indicate that gastrointestinal digestion degrade active peptides from hydrolysates. In contrast with this, Connolly et al. [9] reported that ACE I inhibitory peptides from BSG hydrolysates obtained by Alcalase, Corolase PP, Flavourzyme, and Promod 144MG enzymes retained the activity after simulated gastrointestinal digestion using pepsin and Corolase PP, because of the generation of new peptides with high Pro content. Corolase PP is a porcine pancreatic proteinase preparation with broad specificity containing trypsin, chymotrypsin, and elastase activities [21]. This broad specificity coupled with the high Pro content of the BSG protein extract may result in the release of proline rich peptides. However, AFD and PFD showed lower Pro content than AF and PF (Table 1), which could affect the ACE inhibitory activity after gastrointestinal digestion of peptides. The most effective food protein-derived ACE inhibitory peptides are reported to contain Tyr, Phe, Trp and/or Pro at the C-terminal [6]. The study of inhibition mechanism using Lineweaver-Burk plots showed that AF and PF peptides were competitive inhibitors (ESM 4D). These plots suggest that peptides from AF and PF can bind to the active site to block it or to the inhibitor-binding site that is remote from the active site, but change the enzyme conformation in a way the substrate no longer binds to the active site [22].

Conclusions

Brewers' spent grain peptides from hydrolysates and its gastrointestinal digests delayed thrombin and thromboplastin time respect to the control. However, no effect was observed

on prothrombin time. This result suggested that these peptides inhibit coagulation factors, such as hageman factor (FXIIa), plasma thromboplastin (FXIa), and tenase components (FIXa, FVIIIa, FX, Ca^{2+}, and phospholipids) in the intrinsic blood coagulation pathway. Also exerted their effects on the common pathway by inhibiting the action of thrombin (FIIa), preventing the conversion of fibrinogen (FI) to fibrin (FIa). As far as we know, there is no literature related to the use of brewers' spent grain for obtaining protein hydrolysates with this bioactivity. Moreover, hydrolysates with antithrombotic, α-glucosidase, and α-amylase inhibitory activity increased the bioactivity after gastrointestinal digestion due to the generation of new more active peptides from hydrolysates with low molecular weight, although ACE I inhibition was reduced.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there are no conflicts of interest.

Human and Animal Studies This article does not contain any studies with human or animal subjects.

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