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Purification, identification and *in silico* studies of antioxidant, antidiabetogenic and antibacterial peptides obtained from sorghum spent grain hydrolysate

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ABSTRACT

The identification of antioxidant, antidiabetogenic and antimicrobial peptides obtained from sorghum spent grain (SSG) protein hydrolysate was performed. Antioxidant activity (AOA) was evaluated by $ABTS^{+}$ radical cation scavenging, antidiabetogenic activity (ADA) by dipeptidyl peptidase IV (DPP-IV) inhibition, and antimicrobial activity (AMA) through the minimum inhibitory concentration (MIC) method against 2 Gram-negative and 3 Gram-positive bacteria. SSG peptides were fractionated using a Sephadex G-25 molecular exclusion column, and fractions collected (F1–F6) were evaluated through AOA, ADA and AMA assays. The chromatographic process allowed increasing 20% the AOA and 35% the ADA in F2, and 90% the AMA in fractions F5 and F6. The most active fractions (F2, F5 and F6) were evaluated using LC-ESI-Q-TOF tandem mass spectrometry, which allowed identifying six new peptides with high bioactivity. Moreover, the docking refinement protocol showed that identified peptides could interact with the DPP-IV enzyme, two of them in a competitive inhibition mode. Sorghum spent grain (SSG) could be an important source of bioactive peptides. Additionally, this is the first report indicating the presence of antibacterial peptides encrypted in sorghum proteins.

1. Introduction

Sorghum spent grain (SSG) is the residue obtained after filtering the mash to obtain the sweet wort in the sorghum brewing process, and represents about 85% of the wastes generated in the said industry (Aliyu & Bala, 2011). SSG is lignocellulosic in nature, and contains proteins (20–30 g/100 g dry weight), lignin (~28 g/100 g), hemicelluloses (~25 g/100 g) and cellulose (~17 g/100 g) (Vieira et al., 2014). On the other hand, although the largest production of sorghum beer and, therefore, the generation of SSG are located in Africa, at present there is great global interest in both increasing the variety of beers and generating new beverages suitable for celiacs, which makes sorghum brewing spread around the world (Dabija, Ciocan, Chetrariu, & Codina, 2021). Moreover, SSG is almost identical to the residue obtained by the bioethanol industry in the United States, where they use sorghum as raw material, and the technologies used for brewing and for bioethanol production are very similar (Taylor & Taylor, 2018). For this reason, the study of the use of SSG to generate value-added products could be extrapolated to the bioethanol industry byproduct as long as the use of food grade enzymes and ingredients is considered.

Currently, there is great public pressure to reduce pollution generated by industrial activity. Therefore, the term waste becomes byproduct, which represents a raw material for other processes (Faustino et al., 2019). Besides, SSG has high moisture content, and as drying it is very expensive for the industry, its use as wet waste beyond animal feed would be of great interest for the agribusiness sector. Given its high availability at a low cost, one of the alternatives for using this byproduct is to obtain bioactive peptides from the proteins present in SSG. It has been reported that barley spent grain proteins can be hydrolyzed by proteases, increasing their application and added value (Cian, Garzón, Martínez-Augustin, Botto, & Drago, 2018; Garzón, Cian, Aquino, & Drago, 2020). In the same way, SSG could be used to produce bioactive

Abbreviations: ADA, antidiabetogenic activity; AMA, antimicrobial activity; AOA, antioxidant activity; CFU, colony forming unit; DH, degree of hydrolysis; DPP-IV, dipeptidyl peptidase-IV; F1–F6, fractions from sorghum spent grain hydrolysate; IC50, protein concentration causing an inhibition of 50%; MIC, minimum inhibitory concentration; PH, protein hydrolysate; SSG, sorghum spent grain.

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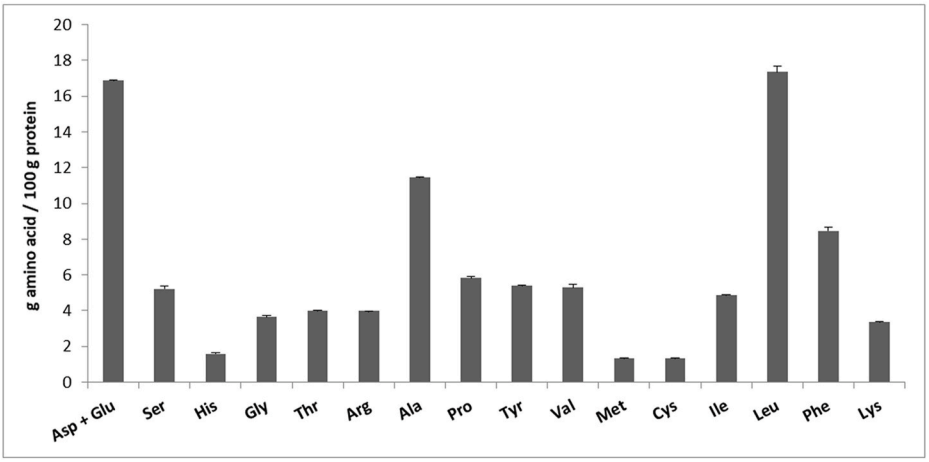

Fig. 1. Amino acid profile of sorghum spent grain.

peptides.

Bioactive peptides are inactive amino acid sequences within the precursor protein ("encrypted"), which exert certain biological functions upon release by proteolysis. The function they exert depends basically on their size, sequence and amino acid composition. Some bioactive peptides have shown to have multifunctional activity, based on their structure and other factors such as hydrophobicity and charge (Görgüç, Gençdağ, &). Antioxidant and antidiabetogenic peptides were found in sorghum kafirin proteins by *in silico* studies (Castro-Jácome, Alcántara-Quintana, & Tovar-Pérez, 2020). This kind of bioactive peptides are strongly influenced by their composition, sequence and molecular mass (Zou, He, Li, Tang, & Xia, 2016).

On the other hand, bacterial resistance to almost all available antibiotics is a major public health problem. Infectious diseases are the second leading cause of death worldwide. In this sense, antimicrobial peptides are considered to have great potential to become a new class of antibiotics to treat resistant bacterial infections (Kumar, Kizhakkedathu, & Straus, 2018). Many studies have shown that enzymatic hydrolysis of food proteins generates potent antimicrobial peptides (Li et al., 2013; Wald, Schwarz, Rehbein, Bubmann, & Beermann, 2016). However, there are no reports of antimicrobial peptides from sorghum proteins.

Bioactive peptides and protein hydrolysates are very promising as valuable functional ingredients in healthy diets to combat the global epidemic of noncommunicable diseases (Chakrabarti, Guha, & Majumder, 2018); therefore, the study of new food sources to obtain these compounds is extremely interesting. As far as we know, there are no reports about characterization of peptides with antioxidant, antidiabetogenic or antimicrobial activity obtained from sorghum spent grain proteins. Accordingly, the aim of this work was to obtain a SSG hydrolysate, and to purify, identify, and perform the *in silico* characterization of peptides responsible for antioxidant, antidiabetogenic and antimicrobial activity.

2. Materials and methods

2.1. Raw material and reagents

Sorghum Spent Grain (SSG) was obtained during the production of ale white sorghum beer (Garzón, Torres, & Drago, 2019). The byproduct was stored at −20 °C until use. Neutral protease-Purazyme® enzyme was provided by Nutring (Buenos Aires, Argentina). Flavourzyme® (9014-01-1) from *Aspergillus oryzae*, dipeptidyl peptidase-IV (DPP-IV, D4943) and Gly-Pro-p-nitroanilide as DPP-IV substrate (G0513) were obtained from Sigma-Aldrich (St. Louis, USA). Other reagents were of analytical grade and obtained from Cicarelli Laboratorios (San Lorenzo, Santa Fe, Argentina).

2.2. Chemical composition and protein amino acid profile of SSG

The contents of moisture and ashes (by gravimetric method), fat (ether extract by the Soxhlet method), proteins (by the Kjeldahl method) and carbohydrates (as the difference from protein, ashes and fat) of SSG were determined according to the standard AOAC methods (AOAC, 2002), and expressed as g/100 g dry basis (d. b.). Amino acid analysis of SSG was carried out according to the method of Alaiz, Navarro, Girón, and Vioque (1992), using a Shimadzu Series LC-20AT pump, with Shimadzu SPDM20A diode array detector, equipped with a 300 × 3.9 mm i. d. reversed-phase column (Novapack C18, 4 μm; Waters). Data were processed using Shimadzu LC solution software. Amino acid content was expressed as g/100 g protein.

2.3. Protein hydrolysate

Protein hydrolysate (PH) was obtained sequentially by Neutral protease-Purazyme® and Flavourzyme® hydrolysis according to the methodology proposed by Cian et al. (2018) for barley spent grain. The hydrolysate was centrifuged at 2000×*g* for 20 min and the supernatant was stored at −20 °C.

The degree of hydrolysis (DH) was calculated determining free amino groups according to Nielsen, Petersen, and Dambann (2001). Protein content was determined according to section 2.2.

2.4. Peptide purification

In order to purify peptides, a fractionation of the PH was performed using a Sephadex G-25 molecular exclusion column (20 × 0.8 cm, Pharmacia Biotech, Sweden). Column void volume was 5 mL (determined with the elution of 1 mL of bovine serum albumin, 1 mg/mL), and column total volume was 35 mL (determined with the elution of 1 mL of Tyr, 0.3 mg/mL).

The PH (1 mL, 50 mg protein/mL) was loaded into the column, previously equilibrated with pH 8.0 100 mmol/L Tris buffer. Elution flow was 0.15 mL/min, and 35 fractions of 1 mL were collected. The absorbance at 280 and 220 nm was determined for each fraction, and the highest absorbance peaks obtained were selected and combined as shown in Fig. 1. Protein content was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). The antioxidant, antidiabetogenic, and antimicrobial activities were evaluated in each selected peak.

2.5. Antioxidant activity (AOA)

To estimate the AOA of PH and their fractions, scavenging of $ABTS^{+}$

radical was evaluated, according to Garzón et al. (2019). The concentration causing an inhibition of 50% (IC_{50}) of $ABTS^{+}$ (0.01 mmol/L in PBS pH 7.4) was determined by serial dilutions from 0 to 6 g protein/L. The experimental data were fitted with the following equation:

$$y = a + b*x$$

where y is the inhibition rate, a and b are the regression parameters, and x is the protein concentration (mg/mL). The IC_{50} value was obtained as:

$$IC_{50} = (y\text{-}50)/b$$

2.6. Antidiabetogenic activity (ADA)

Antidiabetogenic activity (ADA) was evaluated through the inhibition of dipeptidyl peptidase IV (DPP-IV). The experiment was performed in 96-well microplates by measuring the increase in absorbance at 385 nm using Gly-Pro-p-nitroanilide as DPP-IV substrate according to Fontoura et al. (2014). Results were expressed as inhibition percentage of DPP-IV. To determine the IC_{50} of PH and their fractions, serial dilutions from 0 to 15 g protein/L were made. The experimental data were fitted with the following equation:

$$y = y0 + A*\exp(R0*x)$$

where y is the inhibition rate, y_0, A, and R_0 are the regression parameters, and x is the protein concentration (mg/mL). The IC_{50} value was obtained as:

$$IC50 = \ln[(50\text{-}y0/A)]/R0$$

2.7. Antimicrobial activity (AMA)

2.7.1. Microbial cultures

In this study, five test bacterial strains (2 Gram-negative and 3 Gram-positive) were used to assess the AMA of the PH and their fractions. The bacteria used were *Listeria monocytogenes* ATCC 7644, *Salmonella enterica* serotype Enteritidis SE86, *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 1901, and *Bacillus cereus* ATCC 9634. All microbial strains were obtained from the Biochemistry and Applied Microbiology Laboratory, Food Science and Technology Institute, Universidade Federal do Rio Grande do Sul, Brazil.

2.7.2. Inoculum preparation

Bacterial isolates were streaked onto Brain Heart Infusion agar (BHI, Biolab, Hungary) plates and incubated for 24 h at 37 °C. For the AMA assay, colony suspensions in sterile saline (NaCl, 0.0085 g/mL) were adjusted to 10^8 CFU/mL using a standard OD measured at 600 nm (0.600 and 0.150, for *B. cereus* and the rest of the bacteria evaluated, respectively), and were diluted until achieving a final concentration of 10^5 CFU/mL.

2.7.3. Antimicrobial activity

The AMA was evaluated through the minimum inhibitory concentration (MIC) method following the methodology described by Chrysargryis, Xylia, Botsaris, and Tzortzakis (2017), with modifications. For this, 96-well microplates were used. The test was performed by dispersing 45 μL of BHI broth, 45 μL of sample (filtered using sterile 0.22 μm filters), and 10 μL of inoculum at a concentration of 10^5 CFU/mL, to obtain a final concentration of 10^4 CFU/mL. The microplate was covered with a sterile plastic cap and incubated at 37 °C for 24 h with stirring. Microbial growth was confirmed by plating 10 μL of each well at the end of the incubation time, on BHI agar plates. The plates were incubated at 37 °C for 24 h. Positive and negative controls were performed. The positive consisted of adding 45 μL of sterile saline and 10 μL of inoculum to the 45 μL of BHI, and the negative in adding 55 μL of sterile saline to the 45 μL of BHI. The MIC was determined as the lowest concentration of protein (mg/mL) that inhibited 100% growth of test microorganism.

2.8. Identification of peptides using LC-ESI-Q-TOF tandem mass spectrometry

Mass spectrometry analysis were carried out using a Micromass/Waters Q-TOF mass spectrum (Milford, MA, USA) equipped with a nanoelectrospray ionization source (nano-ESI). The instrumental conditions of the mass spectrometer were the following: positive ion mode, nano-ESI flow rate of 0.6 μL/min, sample cone voltage 40 V, capillary 3.3 kV, source temperature 100 °C, gas 5 L/h, and desolvation gas 30 L/h. The Q-TOF was calibrated using 0.1 g/100 mL phosphoric acid in water-acetonitrile (1:1). Data were analyzed with Data Analysis 4.3 software (Bunker, United States). MS/MS data were submitted to the MASCOT server for database searching. The searches were performed against a SwissProtein database. For taxonomy, "*Viridiplantae*" was specified. The probability of random hits (p) was set <0.05, meaning 95% confidence in the correct peptide identification. The peptide mass and the fragment mass tolerance were set at 1.2 Da. The maximum of two missed cleavages was allowed. Also, methionine oxidation was set as a variable modification. Peptide identifications were accepted if they were statistically significant ($p < 0.05$).

2.9. In silico analysis

In silico studies of the obtained peptides were conducted. PepDraw program (http://www.tulane.edu/~biochem/WW/PepDraw/) was used to determine the hydrophobicity. PeptideRanker (http://bioware.ucd.ie) was used to analyze all the peptide sequences obtained. The score obtained was used as an indication of the bioactivity potential. All peptide sequences with score of at least 0.5 were selected for a more thorough complexity analysis. Additionally, the bioactivity estimation of the peptides with a score of at least 0.5 was performed using the BIOPEP-UVW program (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep).

Additionally, determining the structure of protein–peptide complexes is important for understanding the molecular mechanism of related biological processes. In this sense, molecular docking assays were performed using the peptides sequence with predicted DPP-IV inhibitory activity, and a highly known DPP-IV inhibitor peptide was used as a control (IPI, Diprotin A). HPEPDOCK server freely available (http://huanglab.phys.hust.edu.cn/hpepdock/) was used to perform flexible protein-peptide docking. DPP-IV structure was selected from https://www.rcsb.org/in PDB format (PDB entry code: 1PFQ). The best model of each protein-peptide interaction was selected according to the most negative docking score.

The structures obtained were used for the second stage of refinement to find high-resolution modeling of protein-peptide interactions using the FlexPepDock server freely available (http://flexpepdock.furmanlab.cs.huji.ac.il/). FlexPepDock created 200 models for each analysis and they were further ranked based on their Rosetta generic full atom energy score. Three independent replicates for each protein-peptide interaction were analyzed in FlexPepDock server. The best models of each refinement were selected to analyze interactions and binding modes between ligands and DPP-IV. Visual analyses to obtain the interaction points were performed using the PyMol 4.60 software.

2.10. Statistical analysis

Each experiment was performed at least by triplicate. All results were expressed as mean ± SD (standard deviation). Origin 8.0 software (OriginLab, Northampton, MA, USA) was used to perform the curves of IC_{50}. The normal distribution of data was tested using Shapiro–Wilk's test. The homogeneity of variances was tested using Levene's test. One-

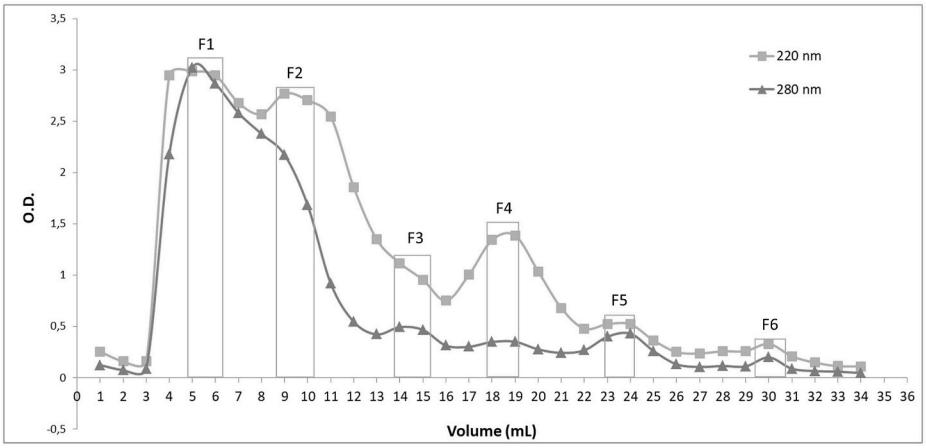

Fig. 2. Elution chromatogram of protein hydrolysate through a Sephadex G-25 molecular exclusion column. F1–F6: fractions selected for analysis. O.D.: optical density.

way analysis of variance (ANOVA) was performed and the statistical differences among samples were determined using the Duncan test. Significance was accepted at $p < 0.05$. Statgraphics Centurion XV 15.2.06 software was used.

3. Results and discussion

3.1. Chemical composition and protein amino acid profile of SSG

The chemical composition of SSG was 6.6 ± 0.2 g water/100 g, 16.3 ± 0.1 g protein/100 g d.b., 5.1 ± 0.0 g fat/100 g d.b., 1.7 ± 0.0 g ash/100 g d.b., and 76.9 ± 0.1 g carbohydrates/100 g d.b. These results show that SSG is rich in protein. In addition, the byproduct obtained could be compared with those of the elaboration of white or red sorghum beer typical of Africa, whose composition in g/100 g d.b was: 19.4–23.4 protein; 3.2–4.5 fat; 43.0–54.0 dietary fiber; 0.8–1.4 ash; 16.7–33.6 carbohydrates (Adewusi & Ilori, 1994). Moreover, the protein content of the native sorghum hybrid used was 9.8 g/100 g d.b. indicating that the mashing process concentrated the protein in SSG. On the other hand, Mussatto, Dragone, and Roberto (2006) suggested that the chemical composition of brewery residues vary depending on the type of grain cultivar used, the harvest time, the malting and maceration processes, and the quality and types of adjuncts added but, in general, it is considered lignocellulosic material, rich in proteins and fibers.

The protein amino acid profile of SSG is shown in Fig. 1. The proteins in this residue are rich in acidic amino acids (Asp and Glu), Ala, Leu, and Phe. SSG contains kafirins (main sorghum proteins), which are rich in glutamine, proline, alanine, and leucine (Taylor & Taylor, 2018).

3.2. Degree of hydrolysis (DH) and protein recovery of SSG hydrolysate

The final DH was 10.9 ± 0.3%. This value was similar to that reported for barley spent grain hydrolysate (DH: 11.4%) obtained using the same hydrolysis system (Cian et al., 2018). However, the percentage of protein recovery (amount of protein solubilized with respect to the initial protein) was 21.9%, higher than that reported by the above mentioned authors (14.5%). The higher protein recovery could be attributed to differences in sorghum and barley prolamins.

3.3. Bioactive properties of PH

Antioxidant peptides are largely studied for their implication in human health improvement through the prevention and treatment of non-communicable chronic degenerative diseases, such as cardiovascular diseases, cancer, or diabetes, among others (Lorenzo et al., 2018). Sorghum PH presented antioxidant activity. The IC_{50} value obtained for ABTS $^{+}$ scavenging assay was 2.79 ± 0.08 mg protein/mL. This value was slightly higher than that obtained by Connolly et al. (2019) for brewer's spent grain protein hydrolysate (~2 mg/mL protein), and by Cian et al. (2018) for *Pyropia columbina* macroalgae protein hydrolysate (2.2 ± 0.1 mg/mL protein) obtained using the same enzymatic system. The AOA of the peptides are strongly influenced by their composition, sequence and molecular mass (Zou et al., 2016). Many of the reported peptides with high capacity to scavenge the radical cation $ABTS^{+}$ have low molecular weight (Cian et al., 2018) since small protein species and free amino acids can easily enter the redox assay system (Segura Campos, Peralta González, Chel Guerrero, & Betancur Ancona, 2013). Moreover, the ABTS method can estimate the antioxidant potential of both hydrophilic and hydrophobic antioxidants; it is simple and fast and does not depend on the medium pH (Lorenzo et al., 2018).

Regarding ADA, the IC_{50} value was 8.47 ± 0.90 mg/mL protein. The DPP-IV enzyme is a serine protease that is expressed in a large number of tissues such as liver, kidney, and small intestine. It has been shown that it indirectly inhibits the secretion of insulin; thus, the inhibitory peptides of this enzyme can be a novel alternative for the treatment of type 2 diabetes (Wang, Yu, Xing, & Li, 2017). Castro-Jácome et al. (2020) performed *in silico* assays over sorghum kafirin proteins and found potential DPP-IV inhibitory peptides. Moreover, brewer's spent grain hydrolysates exhibited 35–85% DPP-IV inhibition at 2 mg/mL proteins (Connolly et al., 2019).

The PH showed growth inhibition against the Gram-positive bacteria *Bacillus cereus* at the protein concentration evaluated (22.5 mg/mL). The MIC of PH against this bacterium (defined as the minimum concentration of an antibacterial agent in effectively inhibiting 100% of bacterial growth) was 11.2 mg/mL protein. Adje et al. (2011) reported that the MIC varies depending on the bacteria tested. Additionally, Daoud et al. (2005) studied the antibacterial activity of peptides fractions obtained from a hemoglobin hydrolysate, and found inhibition only at the concentration evaluated (1 mg/mL) on 4 of the 9 bacteria tested. On the other hand, Hidalgo et al. (2015) studied the antibacterial activity of a casein protein hydrolysate at a concentration of 250 mg/mL against *Listeria monocytogenes, Bacillus cereus, Corynebacterium fimi, Staphylococcus aureus, Salmonella* Enteritidis and *Escherichia coli*, and they found inhibition against 4 of the 6 bacteria evaluated. Additionally, in both studies, there was no inhibition of *B. cereus* by their respective hydrolysates. Moreover, SSG PH was effective against a Gram-positive bacterium, and the lack of inhibition against Gram-negative bacteria could be attributed to the greater complexity of their cell wall structure. Membrane permeabilization is the most widely studied mechanism of antibacterial action. However, there is evidence that some antibacterial peptides exert alternative mechanisms of action at the level of the bacterial cell wall, or at cytosolic level (they would inactivate nucleic acids

Table 1
Antioxidant (ABTS inhibition) and antidiabetogenic activity (DPP-IV inhibition) of peptide fractions (F1–F6) obtained from sorghum spent grain protein hydrolysate by molecular exclusion chromatography (G25).

	ABTS inhibition (%)	DPP-IV inhibition (%)
F1	6.10 ± 0.31^{a}	ND
F2	15.49 ± 0.10^{c}	9.14 ± 0.47^{b}
F3	14.59 ± 0.21^{b}	ND
F4	5.65 ± 0.32^{a}	ND
F5	ND	6.63 ± 0.39^{a}
F6	ND	ND

Media ±standard deviation (S.D.). Samples evaluated at 0.5 mg protein/mL. Different superscript letter in a column means significant differences between samples ($p < 0.05$). ND: not detected.

and cytoplasmic proteins) (Huan, Kong, Mou, & Yi, 2020).

Bacteria have been exposed to antimicrobial peptides for decades, and yet, with the exception of a few species (such as *Burkholderia* spp.), resistance to these peptides has not been reported (Magana et al., 2020). Antimicrobial peptides obtained from natural sources are promising molecules for treating multi-drug resistant microorganisms. In the near future, the benefits of these new compounds will surely be revealed allowing their commercialization (Kumar et al., 2018). On the other hand, *Bacillus cereus* is a biofilm- and spore-forming bacterium that can cause local and systemic infections, including fulminant bacteremia, meningitis, pneumonia, and two types of foodborne diseases due to their toxins: emetic and diarrheal syndrome (Huang, Flint, & Palmer, 2020). This bacterium causes very important economic losses in the food industry, including the dairy industry (Rossi, Gamero Aguilar, Silva, & Centola Vidal, 2018). Therefore, new control strategies and the development of new inhibitor compounds such as additives or food ingredients are necessary.

The hydrolysate of sorghum proteins that remain in the residue after the process of obtaining white sorghum beer could be a source of potent antioxidant, antidiabetogenic, and antimicrobial peptides.

3.4. Peptide fractionation by molecular exclusion chromatography

The elution chromatogram of PH through a Sephadex G-25 molecular exclusion column (1000–5000 Da separation range) is shown in Fig. 2. According to the elution peaks obtained at the absorbances of 220 nm (which detects the peptide bond), and 280 nm (which detects the amino acid residues Trp, Phe and Tyr), fractions F1 to F6 were selected. Considering that the void volume of the column was 5 mL, and that the total volume was 35 mL, the F1 and F6 fractions would correspond to peptides of molecular size greater than 5000 Da, and smaller than 1000 Da, respectively, while F2–F5 would be within the range of column separation.

Although molecular exclusion chromatography is a time-consuming and expensive separation technique, it is highly selective, and of very good resolution, which has been applied to the separation and purification of peptides for years (Wang, Yu, Xing, & Li, 2017).

3.5. Antioxidant and antidiabetogenic properties of peptide fractions

Inhibition percentages of the $ABTS^{+}$ radical and the DPP-IV enzyme of the different fractions evaluated are shown in Table 1. At the protein concentration studied (0.5 mg/mL), only 4 of the 6 fractions exhibited AOA, while only 2 fractions presented ADA. On the other hand, F2 was the fraction with the highest inhibition activity in both properties evaluated.

Although the dominant molecular weight of most of the AOA peptides reported in the bibliography is less than 1000 Da, and considering the fact that they contain between 3 and 6 amino acid residues (Zou et al., 2016), some studies have reported an inverse relationship between DH and $ABTS^{+}$ radical scavenging activity (Alemán, Giménez, Pérez-). In this work, the more active $ABTS^{+}$ inhibitory peptides of PH presented a molecular weight closer to 5000 Da. In addition, they could have hydrophobic residues that would generate a cluster of peptides during elution, which would cause them to migrate as larger molecules. In this sense, it has been observed that peptide hydrophobicity is a very important factor of AOA, since it increases accessibility to hydrophobic target compounds such as fatty acids (García, Puchalska, Esteve, & Marina, 2013).

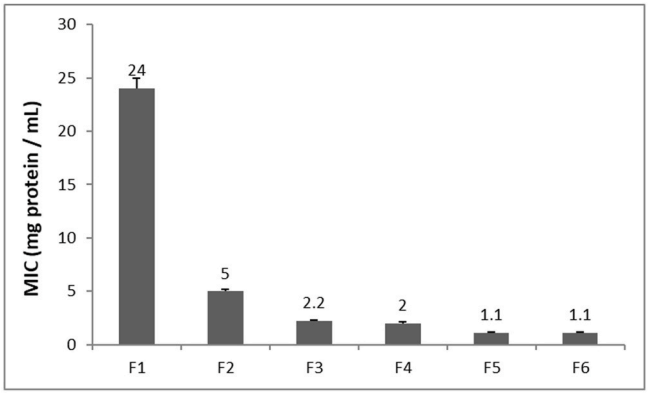

Fig. 3. Minimum inhibitory concentration (MIC) against *Bacillus cereus* of the F1–F6 fractions obtained from protein hydrolysate by molecular exclusion chromatography.

On the other hand, the length of DPP-IV inhibitory peptides is not critical for such bioactivity, since both di-peptides and oligopeptides greater than 10 residues exhibited potent activity (Nongonierma & Fitzgerald, 2014). Thus, it would be feasible that the eluted fractions of the G-25 column with different molecular size have DPP-IV inhibitory activity.

The F2 IC_{50} values of $ABTS^{+}$ and DPP-IV inhibition were 2.27 ± 0.03 and 5.49 ± 0.15 mg protein/mL, respectively. Taking into account that a lower IC_{50} value indicates that a lower protein concentration is able to inhibit 50% of the radical or enzyme, fractionation of PH allowed concentrating the ABTS scavenging at approximately 20%, and the DPP-IV inhibition potency at 35%, in fraction F2.

Antioxidant and antidiabetogenic peptides obtained from PH could be added into functional foods, considering that bioactive peptides have several features that make them suitable for use as therapeutic agents. These traits include (a) high biospecificity for the target molecules; (b) high activity and broad spectrum of action, including some individual peptides with multifunctional properties; (c) low toxicity and lower incidence of accumulation in body tissues, compared to small molecules; (d) high structural diversity; (e) small size (compared to antibodies), which facilitates its transport, and implies that these peptides have a low probability of triggering undesirable immune responses (Agyei, Ongkudon, Yi Wei, Chan, & Danquah, 2016). However, further studies about toxicity or accumulation in body tissues, among others, need to be performed for using SSG bioactive peptides as therapeutic agents.

3.6. Antimicrobial property of peptide fractions

Fig. 3 shows the results obtained from MIC of the F1–F6 fractions obtained from PH by molecular exclusion chromatography. All of them presented antibacterial activity against the Gram-positive bacterium *Bacillus cereus* ATCC 9634. The antibacterial potency increased as the elution volume increased. In this sense, sorghum PH peptides with smaller molecular size would have a greater ability to inhibit the bacteria studied. Due to the fractionation, the antibacterial peptides were concentrated in F5 and F6, increasing the capacity by approximately 90%, compared with PH (MIC = 11.2 mg/mL).

Although antibacterial peptides are generally long peptides of 12–50 amino acid residues, the presence of short peptides with high

Table 2
Identification of peptides in fractions F2, F5 and F6 obtained from the molecular exclusion chromatography and *in silico* analysis.

	Sequence	Mass (Da)	Hydrophobicity (Kcal/mol)[a]	Peptide Ranker Score[b]	Potential Bioactivity[c]
F2	GAGGGAK	516.26	+16.30	0.3035	
	GGAAGGR	544.27	+15.31	0.5083	Antioxidative/DPP-IV inhibitor
	DGAAAGP	557.24	+15.48	0.3599	
	DGLGAVG	587.29	+13.78	0.2462	
	KGGGGPK	599.34	+18.24	0.4583	
	GAGGGHR	610.29	+17.14	0.4778	
	EQDRR	702.34	+19.56	0.1569	
	PPPGSKSYGT	989.48	+13.88	0.5268	DPP-IV inhibitor
	AGLPTEEKPPLL	1263.70	+16.53	0.5556	Antioxidative/DPP-IV inhibitor
	QADPKTFYGLM	1269.60	+12.81	0.8869	DPP-IV inhibitor
	GPPKVAPGKDISASFGGEWL	2012.03	+21.08	0.8928	Antioxidative/DPP-IV inhibitor
F5	AAGGAAF	563.26	+10.49	0.6790	DPP-IV inhibitor
	MAAALAR	702.38	+9.79	0.3252	
F6	GGAIAPA	555.30	+10.72	0.3535	
	LQGGGGL	600.32	+10.77	0.4793	

[a] Obtained with *PepDraw* Program;
[b] Obtained with *distilldeep PeptideRanked* Program;
[c] Obtained with *BIOPEP-UVW* tools;

antimicrobial potency obtained by enzymatic hydrolysis has been reported. Adje et al. (2011) studied the antibacterial activity of peptides from a hemoglobin hydrolysate. They reported that both small (668.8 Da) and large (3900.5 Da) peptides presented MICs of the order of μmol/L (high antibacterial potency) against the bacteria *Micrococcus luteus*, *Listeria innocua*, and *Escherichia coli*. Additionally, Jang, Jo, Kang, and Lee (2008) demonstrated that short peptides of 8 (GLSDGEWQ) and 6 residues (DFHINQ) inhibited the growth of *Bacillus cereus* at a concentration of 400 μg/mL.

Many studies have shown that the enzymatic hydrolysis of milk, egg or meat proteins generates potent antimicrobial peptides (Hidalgo et al., 2015; Memarpoor-Yazdi, Asoodeh, & Chamani, 2012; Thammasirirak et al., 2010; Wald et al., 2016). However, only a few studies have focused on obtaining this type of bioactive compounds from cereals and the residues obtained from their processing (Amadou, Le, Amza, Sun, & Shi, 2013; Li et al., 2013). Additionally, to date there are no reports indicating the presence of antibacterial peptides in sorghum proteins. On the other hand, antibacterial peptides of short chain length and simple amino acid composition could be more favorable in reducing production costs and in facilitating pharmaceutical optimization (Amadou et al., 2013).

3.7. Identification of peptides and in silico analysis

In order to characterize the molecular mass and amino acid sequence of the peptides from the fractions with higher activity (F2, F5 and F6), an analysis by LC-ESI-Q-TOF tandem mass spectrometry was performed. A search for MS/MS fragments in MASCOT allowed identifying the peptides shown in Table 2. The peptides identified were statistically significant ($p < 0.05$), thereby confirming their identity with 95% confidence. As mentioned above, the fraction with the highest AOA and ADA obtained had a MW around 4000 Da. However, the peptide identified from F2 after the MS/MS analysis showed a smaller size (516.26–2012.03 Da). This may be due to hydrophobic interactions between the peptides, which generate large aggregates that are not resolved in molecular exclusion chromatography. Note that this chromatographic process was carried out under native conditions, and the hydrophobic values reported in Table 2 for peptides found in the F2 fraction confirmed the hydrophobicity of these peptides. In agreement with this outcome, Prados, Marina, and García (2018) found that the most active fraction of inhibitory peptides obtained from the ultrafiltration process had a MW higher than 5000 Da. However, they reported that the active peptide had a smaller size (700–1500 Da) according to the MS/MS analysis.

PeptideRanker software was employed to assign a score of bioactivity probability to each sequence. For every peptide, PeptideRanker predicts the probability (between 0 and 1) of that peptide being bioactive. The closer the predicted probability is to 1, the more confident the PeptideRanker is that the peptide is bioactive, and a score higher than 0.5 could be considered a good probability of bioactive potential. In this sense, five peptides found in the F2 fraction and only one peptide found in F5 could be bioactive. Moreover, the analysis of potential bioactivity performed with BIOPEP-UVW (Table 2) over those peptides with a score higher than 0.5 revealed that in F2 all of the five peptides analyzed could have DPP-IV inhibitory activity, while only three identified peptides could have antioxidant activity (GGAAGGR, AGLPTEEKPPLL, GPPKVAPGKDISASFGGEWL).

It has been shown that Pro (P) and Ala (A) residue-rich proteins can generate peptides with high inhibitory capacity of DPP-IV (Wang, Yu, et al., 2017). Moreover, Pro at the P1- or P2- position is the preferred amino acid residue, and Alanine (A), Glycine (G), and other small residues are also accepted by the hydrophobic pocket of DPP-IV. At the P2-position, various hydrophobic, basic, or neutral amino acid residues, or amino acid residues with bulky side chains may enhance the binding ability (Rivero-Pino, Espejo-Carpio, & Guadix, 2020).

On the other hand, a study performed with 42 antioxidant peptides from natural proteins showed that the most abundant amino acid residues were Gly (G), Pro (P) and Leu (L), followed by Ala (A), Tyr (F) and Val (V) (Zou et al., 2016). Considering the most frequent amino acids found in F2 antioxidant peptides, Ala and Pro possess scavenging activity for free radicals, while Gly may be responsible for forming a favorable hydrophobic micro-environment for peptide molecules (Zou et al., 2016). Moreover, high proportion of hydrophobic amino acids has been reported in peptides with high antioxidant activity (Lorenzo et al., 2018). Additionally, plant-derived bioactive peptides demonstrate great potential against multiple diseases since they have two or more bioactivities (Tok, Moulahoum, Kocazorbaz, & Zihnioglu, 2021).

Regarding the most active fractions for *Bacillus cereus* inhibition (F5 and F6), only four peptides could be identified. One of them in F5 (AAGGAAF) also presented probable DPP-IV inhibition potential. As shown before (Table 1), F5 was one of the fractions that presented DPP-IV inhibition potential. On the other hand, the sequence obtained in fractions F5 and F6 were predicted in new antibacterial peptide potential database tools (https://dbaasp.org/), and none of them could be antibacterial peptide. In this sense, the peptides responsible for *Bacillus cereus* inhibition could not be identified. Mikut et al. (2016) analyzed a

Table 3
Interaction energies obtained from molecular docking.

	Docking score[a]	Rosetta Score[b]	Interface Energy (Kcal/mol)[b]
GGAAGGR	−154.837	−924.627	−15.851
PPPGSKSYGT	−223.669	−743.516	−16.669
AGLPTEEKPPLL	−166.990	−911.735	−14.981
QADPKTFYGLM	−219.312	−930.227	−18.409
GPPKVAPGKDISASFGGEWL	−235.975	−928.110	−16.082
AAGGAAF	−168.063	−922.523	−15.825

[a] Peptide-DPPIV docking using HPEPDOCK server;
[b] Structure refinement using FlexPepDock server.

library with short antimicrobial peptides (3000 peptides) and found the importance in the balance between positive charge (Arg or Lys), hydrophobicity and content of tryptophan against growth inhibition of Gram (−) and Gram (+) bacteria. It could probably occur that both in fractions F5 and F6, short peptides with positive charge and hydrophobic residues act against *B. cereus*. Further studies are needed to confirm this hypothesis.

3.8. DPP-IV–peptide docking

The six peptides with potential DPP-IV inhibitory activity from F2 and F5 (GGAAGGR, PPPGSKSYGT, AGLPTEEKPPLL, QADPKTFYGLM, AAGGAAF, and GPPKVAPGKDISASFGGEWL) were subjected to docking against the DPP-IV enzyme (PDB code: 1PFQ) using the HPEPDOCK server. DPP-IV comprises some pockets where ligands can "fit". Recently, it has been reported that there are three binding pockets: S1 consists of Tyr547, Ser630, Tyr631, Val656, Trp659, Tyr662, Asn710, Val711, and His740; S2 consists of Glu205, Glu206, and Tyr662; and S3 consists of Ser 209, Arg358 and Phe357 (Kim et al., 2018). Moreover, Metzler et al. (2008) reported Ser630, His740, and Asn708 as the catalytic triad.

The docking energy scores are shown in Table 3. Theoretically, peptides that are fixed to one pocket of the reaction center with lower affinity energy are more likely to be the inhibitors and vice versa. The docking score of Diprotin A (IPI) with DPP-IV enzyme was −101.680. In this sense, taking into account that Diprotin A has been reported as the most potent DPP-IV inhibitory peptide (IC_{50} = 5 μmol/L) (Rivero-Pino et al., 2020), docking scores of SSG peptides were favorable compared to the control.

In a second stage, a high-resolution structural and energy refinement of the peptide in the binding site interaction was performed using the FlexPepDock server. Rosetta score and interface energies were extracted (Table 3). The negative values obtained confirm that the interaction of evaluated ligands and the DPP-IV enzyme was possible and favorable for all the peptides analyzed. An explanation for why DPP-IV can accept inhibitors of various shapes is that this enzyme has a large cavity (diameter ≥20 Å), which allows the approach of these inhibitors to its active site (Nojima et al., 2016).

The interaction points between peptides and residues from DPP-IV enzyme pockets (S1, S2 or S3) are shown in Fig. 4. All the interactions displayed are lower than 3.0 Å. Firstly, it can be observed that one peptide could interact with residues of two or more pockets. This is probably due to both the large sequence of peptides analyzed and the proximity between the residues from different pockets (Nojima et al., 2016). Moreover, PPPGSKSYGT and QADPKTFYGLM peptides interact with two residues of the active site (Ser630, His740), so the interaction could be competitive, while all other peptides would interact in a non-competitive mode.

4. Conclusions

Sorghum spent grain (SSG) could be an important source of bioactive peptides. The present data demonstrate that the enzymatic hydrolysis of SSG allowed obtaining a hydrolysate with ABTS scavenging activity, DPP-IV inhibition potential, and *Bacillus cereus* inhibition growth. Moreover, fractionation by molecular exclusion chromatography allowed concentrating the bioactivity potential.

This is the first report indicating the presence of antibacterial peptides in sorghum proteins. Moreover, new peptides sequences with

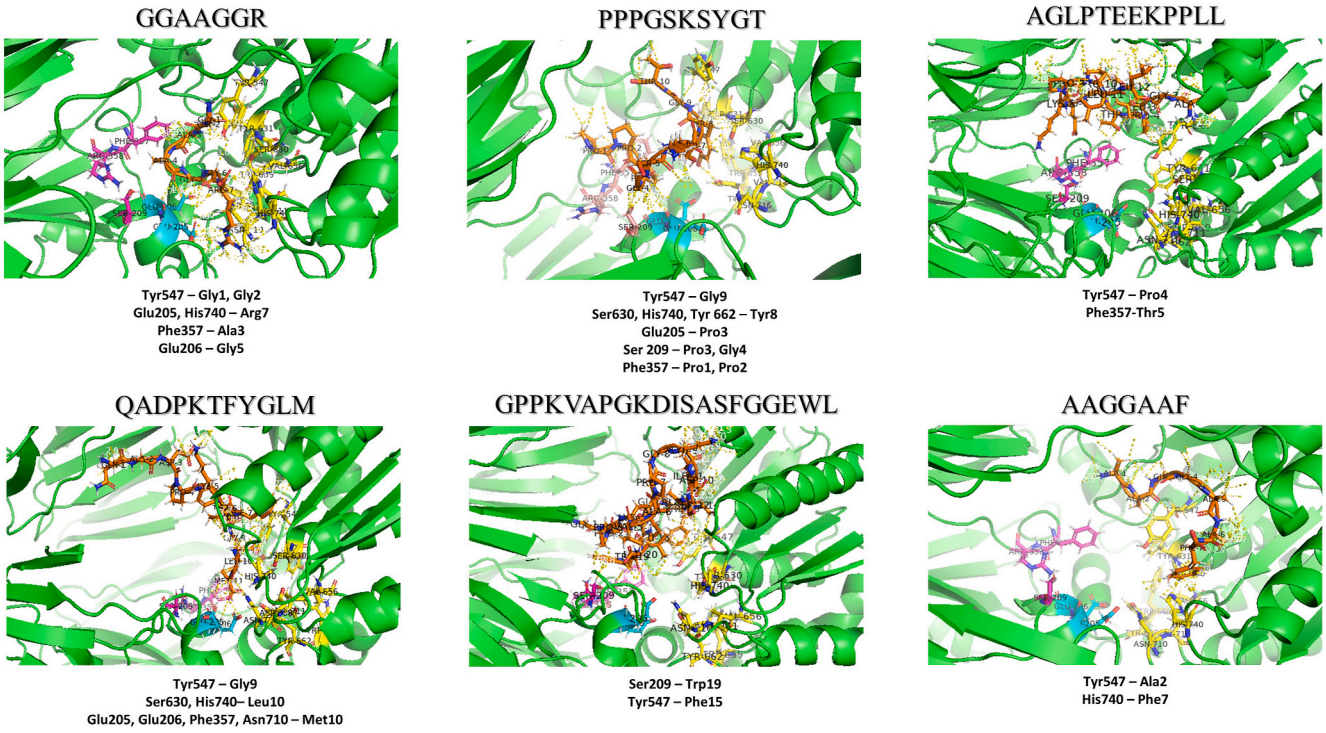

Fig. 4. Interaction points between peptides and DPP-IV enzyme. Orange: Peptide; Yellow: S1 pocket residues; Blue: S2 pocket residues; Pink: S3 pocket residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

antioxidant and antidiabetogenic activity were reported. It was demonstrated that the peptides obtained with higher PeptideRanker score could interact with the DPP-IV enzyme, two of them in a competitive inhibition mode (PPPGSKSYGT and QADPKTFYGLM).

Although it was possible to obtain the sequence of peptides with antioxidant and antidiabetogenic potential, further studies including their chemical synthesis and *in vitro* and *in vivo* assays, should be carried out to confirm their bioactivity and mechanism of action.

Credit author statement

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AGG and FFV carried out the experiments. AGG, FFV, AB and SRD analyzed the data and wrote the paper, and had primary responsibility for final content. All authors read, edit and approved the final manuscript.

Contribution of authors

AGG and FFV carried out the experiments. AGG, FFV, AB and SRD analyzed the data, wrote the paper and had primary responsibility for the final content. All authors read and approved the final manuscript.

Declaration of competing interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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