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Isolation and identification of cholesterol esterase and pancreatic lipase inhibitory peptides from brewer's spent grain by consecutive chromatography and mass spectrometry

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Abstract

The isolation and identification of cholesterol esterase (CE) and pancreatic lipase (PL) inhibitory peptides obtained from brewer's spent grain (BSG) protein hydrolysate was performed. BSG peptides were fractionated and purified sequentially by anionic exchange, gel filtration (FPLC), and reversed phased-high performance liquid chromatography (RP-HPLC). Fractions from each chromatography step were collected and *in vitro* enzyme inhibitory activity was evaluated. Chromatographic purification process allowed concentrates the *in vitro* activities. The most active fractions were evaluated using MALDI-TOF tandem mass spectrometry, which allowed identified three peptides: a peptide with the highest CE inhibition capacity (WNIHMEHQDLTTME) and two peptides with PL inhibition capacity (DFGIASF and LAAVEALSTNG). The three peptides found showed hydrophobic and acidic amino acid residues (Asp and Glu), and/or their amines (Asn and Gln), which could be a common feature among lipid-lowering peptides related to CE and PL enzyme inhibition. *In silico* studies showed that the three peptides had high hydrophobicity and were susceptible to enzymatic hydrolysis performed by trypsin, pepsin, and pancreatin. BSG byproduct was a good source of CE and PL inhibitory peptides, adding value to this byproduct of beer industry. This is the first report to demonstrate that BSG peptides are able to inhibit CE and PL enzymes.

Keywords: cholesterol esterase inhibition; pancreatic lipase inhibition; brewer's spent grain; chromatography process; MALDI-TOF.

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1. Introduction

Brewers' spent grain (BSG) is the most abundant by-product generated from the beer-brewing process. This residue denotes 85% of the total byproducts generated in the beer industry.[1] The main constituents of BSG include fiber (30–50% w/w) and protein (19–30% w/w).[2] For every 10 liters of beer produced, 2 kg of BSG are generated.[3] Thus, BSG merits considerable attention, not only for the large amounts produced, but also due to its valuable protein content. In this regard, one of the alternatives for using this byproduct is to obtain bioactive peptides from the residual proteins.[4] It has been reported that BSG peptides can exert different *in vitro* bio-functional properties such as: antioxidant, anti-microbial, anti-inflammatory, hypoglycaemic, antithrombotic, and angiotensin converting enzyme I (ACE-I) inhibitory activities.[4-6] However, as far as we know, there is no research about the effect of BSG peptides on cholesterol esterase and pancreatic lipase inhibitory activities and even less about the purification and identification of these peptides.

Hyperlipidemia is an abnormal health condition characterized mainly by elevated levels of triglycerides and serum cholesterol, called hypertriglyceridemia and hypercholesterolemia, respectively.[7] The prevalence of hyperlipidemia is a risk factor in cardiovascular diseases such as coronary heart disease and atherosclerosis. Different synthetic drugs have been developed to treat this disease. However, various side effects have been described derived from long-term use of these synthetic drugs.[8,9] Therefore, there is an increased interested to developed new natural lipid-lowering substances.[9] In this regard, BSG proteins have high content of hydrophobic amino acids.[10] Thus, it could be a good source of peptides with lipid-lowering properties.[9] The aims of this work were to evaluate the capacity of BSG protein hydrolysate to inhibit cholesterol esterase (CE) and pancreatic lipase (PL), and to isolate and identify the novel peptides

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responsible for these activities. Furthermore, peptides characterization through *in silico* analysis was performed.

2. Material and Methods

2.1. Raw material and reagents

Brewers' spent grain (BSG) was supplied by Santa Fe® Brewery (Santa Fe, Argentina). Neutral protease-Purazyme® enzyme was provided by Nutring (Buenos Aires, Argentina). Flavourzyme® (9014-01-1) from *Aspergillus oryzae* was obtained from Sigma-Aldrich (St. Louis, USA). Other reagents were of analytical or HPLC grade, and obtained from Cicarelli Laboratorios (San Lorenzo, Santa Fe, Argentina).

2.2. Protein hydrolysate

Protein hydrolysate was obtained sequentially by Neutral protease-Purazyme® and Flavourzyme® hydrolysis according to Cian *et al*.[10] Protein content was determined by AOAC methods,[11] resulting 49.1 g protein/100 g b.s. The degree of hydrolysis (DH) was 14.30 ± 0.01% and was measured according to Nielsen *et al*.[12]

2.3. Cholesterol esterase and pancreatic lipase enzyme inhibition

Cholesterol esterase and pancreatic lipase enzyme inhibition methods, which measure the ability of peptides to reduce the absorption of dietary cholesterol and triglycerides, respectively, were performed by triplicate.

2.3.1. Cholesterol esterase (CE) enzyme inhibition

The method of CE inhibition was performed following the methodology proposed by Prados *et al*.[9] with some modifications. Briefly, sample (100 µL) was pre-incubated for

10 min at 37°C together with the substrate (*p*-nitophenyl palmitate, pNPP, 20 mM, 10 µL), phosphate buffer (100 mM with 100 mM NaCl pH 7.0, 580 µL), taurocolic acid (12 mM, 150 µL), and acetonitrile (40 µL). Subsequently, the pancreatic CE enzyme (Wiener Lab) (100 µL) was added and incubated for 60 min at 37°C. Product formation was determined spectrophotometrically at 405 nm. In addition, a control of maximum enzymatic activity (without sample), and a sample blank (without substrate) was performed.

2.3.2. Pancreatic lipase (PL) enzyme inhibition

The method of PL inhibition was performed following the methodology proposed by Prados *et al.*[9]

2.4. Isolation and identification of cholesterol esterase and pancreatic lipase inhibitory peptides

BSG peptides were fractionated and purified sequentially by anionic exchange, gel filtration (FPLC), and reversed phased-high performance liquid chromatography (RP-HPLC).

2.4.1. Fractionation by anionic exchange chromatography

Taking into account that the charge is one of the main characteristics of the peptide capacity to inhibit these types of enzymes, a first separation and purification step was carried out using an ionic exchange chromatography, in order to analyze the inhibition activity based on the charge of the peptides. The BSG hydrolysate was fractionated by anion exchange chromatography at pH 7.0 according to Gomez *et al.*[13] For this, an AG-X4 resin (100 - 200 mesh) - Biorad® was used. Elution was performed with a linear

gradient of NaCl (0.0 mol/L, 0.2 mol/L, 0.4 mol/L, and 0.6 mol/L), which allow obtaining four fractions (***A1***, ***A2***, ***A3*** and ***A4,*** respectively).

The protein content of each fraction was determined by the method proposed by Lowry *et al.*[14] The CE and PL enzyme inhibition activities were determined *in vitro* as was described before at 4 mg protein/mL.

The most bioactive fractions were analyzed according to their protein amino acid profile following the method of Alaiz *et al.,*[15] 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. Moreover, the total mEq of hydrophobic (Gly, Ala, Val, Leu, Ile, Met, Phe, Trp, and Pro), acid (Glu and Asp), and basic amino acids (Lys, Arg, and His) was calculated. The analysis was performed by triplicate.

2.4.2. Fractionation by gel filtration chromatography using FPLC

The most bioactive fractions obtained by anion exchange chromatography (***A1*** and ***A3)*** were selected to continue the purification process by FPLC according to Cian *et al.*[6] This stage was carried out with the aim of separating the bioactive peptides based on their molecular size, and therefore analyzing how this factor affected the inhibition properties of CE and PL enzymes. To perform this chromatography step, an AKTA Prime system equipped with a Superdex 75 (GE Life Sciences, Piscataway, NJ, USA) was used. Elution was monitored at 280 nm and molecular mass was estimated using molecular weight standards from Pharmacia Fine Chemicals (Piscataway, NJ, USA): conalbumin (75000 Da), carbonic anhydrase (29000 Da), cytochrome C (12500 Da), aprotinin (6512 Da), bacitracin (1450 Da), cytidine (246 Da) and glycine (75 Da).

The protein content from FPLC fractions was determined by the method proposed by Lowry *et al.*[14]

The fractions obtained by FPLC from ***A1*** were named: ***B1, B2, B3, B4, B5, B6*** and ***B7***. These fractions were subjected to the assay of CE enzyme inhibition at 0.4 mg protein/mL. On the other hand, the fractions obtained by FPLC process from ***A3*** were named: ***b1, b2, b3,*** and ***b4*** and subjected to the assay of PL enzyme inhibition at 1.4 mg protein/mL

2.4.3. Fractionation by reversed phased - high performance liquid chromatography (RP-HPLC)

The most bioactive fractions obtained from FPLC process (***B3*** and ***b4)*** were selected to continue the purification process by RP-HPLC. Taking into account that hydrophobicity is a key property in these types of peptides, the objective of performing an RP-HPLC step was to separate and purify the most bioactive peptides based on their hydrophobicity, and to obtain a sample as pure as possible for the identification of the peptides by mass spectrometry.

The RP-HPLC process was performed according to Zhang *et al.*[16] with modifications. For this, a Phenomenex C18 column (Gemini 250 x 4.6 mm, 5µm) was used. Elution was performed by gradient, using as mobile phase A: water with 0.1% trifluoroacetic acid (TFA), and as mobile phase B: acetonitrile with 0.1% TFA. The gradient increased linearly, from 0% to 40% of B, in a run time of 60 min. The column was placed at a temperature of 40°C, and the elution flow was 1 mL/min. Fractions that gave absorbance peaks at 220 nm and/or 280 nm were collected. The fractions obtained were listed C1-Cn.

For each fraction obtained, the protein content was determined by measuring sample absorbance at 280 nm.

The fractions obtained by RP-HPLC process from ***B3*** were named: ***C1***, ***C2***, ***C3***, and ***C7***. These fractions were subjected to the assay of CE enzyme inhibition at 0.23 mg protein/mL. On the other hand, the fractions obtained from ***b4*** were named: ***c1*** and ***c2*** and subjected to the assay of PL enzyme inhibition at 0.06 mg protein/mL.

The most bioactive RP-HPLC fractions were further selected to identify the peptides responsible for each activity using MALDI-TOF tandem mass spectrometry.

2.5. Identification of peptides using MALDI-TOF tandem mass spectrometry

A sample volume (0.5 µl) with equal amount of the matrix (alpha-cyano-4-hydroxy cinnamic acid) was spotted onto a MALDI plate. The plates were allowed to dry at room temperature. The MS/MS analysis was performed on the AXIMA-iD Plus (Shimadzu) equipment. The determinations were made in reflectron mode. The MS/MS data were submitted to the MASCOT server for database searching. The searches were performed against a NCBI protein 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 ($p<0.05$).

2.6. *In silico* analysis

Studies *in silico* of the peptides obtained were conducted. The hydrophobicity was determined using the *PepDraw* program (http://www.tulane.edu/ ~biochem/ WW

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/PepDraw/), and a simulation of the gastrointestinal digestion of the peptides was performed using the *BIOPEP-UVW* program (http://www.uwm.edu.pl/ biochemia / index.php / pl / biopep).

Additionally, protein-peptide dockings were performed. In a first stage, CABS-dock server freely available (http://biocomp.chem.uw.edu.pl/CABSdock) was used to perform flexible protein-peptide docking. Enzymes structures were selected from https://www.rcsb.org/ in PDB format (PDB entry codes: 2BCE and 1GPL, for CE and PL enzymes, respectively). For each docking, 50 cycles of Monte Carlo simulation was performed. The best model of each protein-peptide interaction was selected according to average root mean square deviation (RMSD) values. Moreover, contact map (interaction interface between the peptide and the receptor residues) was analyzed with a cut off of 4.5 Å.

The structures obtained from this analysis with CABS-dock server 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. Interface energy was extracted from Rosetta score.

2.7. Statistical analyses

Results were expressed as the mean ± standard deviation and were analyzed by analysis of variance (ANOVA). The statistical differences among samples were determined using the least significant difference (LSD) test with a level of signification $\alpha = 0.05$.,

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using the STATGRAPHICS Centurion XV 15.2.06 (StatPoint Technologies, Inc., Warrenton, Virginia, USA).

3. Results and Discussion

3.1. Cholesterol esterase and pancreatic lipase inhibitory activity of BSG hydrolysate

The BSG hydrolysate had a good lipid-lowering activity evaluated through the inhibition of lipid metabolism related enzymes such as cholesterol esterase (CE) and pancreatic lipase (PL). The inhibitory activity on CE and PL enzymes at 4 mg protein/mL were 33.5 ± 1.2% and 17.8 ± 1.7%, respectively. To date, this is the first work that reported peptides from BSG with this *in vitro* activity. Thus, the hydrolyzate was used as starting source to isolate and identify peptides responsible for these bioactive properties.

3.2. Isolation of cholesterol esterase and pancreatic lipase inhibitory peptides

3.2.1. Cholesterol esterase and pancreatic lipase inhibitory activities from anionic exchange chromatography fractions

The first purification step consisted in anion exchange chromatography. For the elution process, a linear gradient of sodium chloride was used, obtaining four fractions: ***A1***, ***A2***, ***A3*** and ***A4***. The first one (***A1***) was eluted with distilled water (it was not retained by the column), while the others were obtained with 0.2, 0.4 or 0.6 mol/L NaCl, respectively.

As shown in **Figure 1a**, the fraction ***A1*** had the highest inhibitory activity on CE enzyme ($p < 0.05$). The peptides present in ***A1*** could have a higher proportion of basic amino acids generating a positive overall charge density because of the column used

exchanges anions. In this sense, it has been reported that the basic amino acids play an important role in the primary structure of the CE enzyme inhibitor peptides.[17]

Regarding PL enzyme inhibition, ***A3*** fraction showed the highest inhibitory activity (**Figure 1b**). This result could be due to presence of peptides with a high proportion of negatively charged amino acids. It has been reported that strong electrostatic interactions between the peptides and the catalytic residues of PL favor their inhibition.[18] In this regard, Prados *et al*.[9] observed that peptides obtained from olive seed protein hydrolysate with good inhibitory activity on PL had a high proportion of negatively charged amino acids.

Additionally, it is important to highlight that the fractionation by anionic exchange chromatography concentrated the inhibition activity in 1.4 and 2.3 times for CE and PL enzymes, respectively.

Figure 2 shows the amino acid profile of the most bioactive fractions (***A1*** and ***A3***). For both, the content of hydrophobic amino acids was high, it being 58 and 54% for ***A1*** and ***A3***, respectively. This is a consistent result since hydrophobicity plays a fundamental role in lipid-lowering activity evaluated *in vitro*.[9] Moreover, ***A1*** presented a relatively high proportion of basic amino acids (15%), while ***A3*** exhibited a relatively high content of acidic amino acids (11%). These results agree with the above, indicating that ***A1*** had hydrophobic peptides with a positive charge density, while ***A3*** present hydrophobic peptides with a negative charge density.

3.2.2. Cholesterol esterase and pancreatic lipase inhibitory activities from FPLC fractions

Fractions ***A1*** and ***A3*** were analyzed and fractionated by FPLC gel filtration **(Figure 3)**. The fractions obtained from ***A1*** were named ***B1***, ***B2***, ***B3***, ***B4***, ***B5***, ***B6*** and ***B7,*** and subjected to the assay of CE enzyme inhibition. On the other hand, the fractions obtained by FPLC process from ***A3*** were named ***b1***, ***b2***, ***b3***, and ***b4,*** and assayed by PL enzyme inhibition.

As can be seen, ***A1*** presented four main peaks: >70000 Da, ~8000 Da, 2100 Da, and 450 Da (**Figure 3a)**. The components higher than 70000 Da presented an elution volume higher than that corresponding to exclusion volume, while the ~8000 Da peak could correspond to polypeptides. The peaks of 2100 Da and 450 Da would be intermediate MW species and low MW peptides, respectively. Additionally, ***A1*** profile showed a shoulder of ~1000 Da, which can be attributed to the presence of low MW peptides. Note that 2100 Da peak represented 40% of the total area of the chromatogram. If the average size of an amino acid is considered to be 120 Da, ***A1*** would have a high proportion of oligopeptides.

Regarding FPLC gel filtration profile of ***A3*** fraction (**Figure 3b**), three main peaks were observed: 3300 Da, 1700 Da, and 70 Da. The peaks of 3300 Da and 1700 Da could correspond to intermediate MW species, while 70 Da peak correspond to free amino acids. Additionally, ***A3*** profile showed a shoulder of ~1000 Da, which can be attributed to the presence of low MW peptides. It is noteworthy that the peak of 1700 Da represented 37% of the total area of the chromatogram, which would indicate that ***A3*** has a high proportion of intermediate MW species.

As shown in **Figure 4a**, all the fractions obtained from ***A1*** by FPLC process (***B1***, ***B2***, ***B3***, ***B4***, ***B5***, ***B6*** and ***B7***) inhibited the CE enzyme. The most active was ***B3***, indicating that polypeptides with MW higher than 8000 Da would be responsible for this

inhibitory activity. These results agree with that obtained by Prados *et al.*,[9] who reported that the highest CE inhibitory activity was found in fractions with MW higher than 5000 Da obtained by ultrafiltration. Moreover, they found that fractions lower than 5 kDa had the lowest inhibition values. Note that CE inhibitory activity of ***B3*** was increased 8 times respect to ***A1***.

On the other hand, all the fractions obtained from ***A3*** by FPLC process (***b1***, ***b2***, ***b3***, and ***b4***) inhibited the PL enzyme (**Figure 4b**). The most active fraction was ***b4***, indicating that low MW peptides ~1000 Da would be responsible for this inhibitory activity. In agreement with this results, the peptides reported to date with the ability to inhibit PL enzyme showed molecular size between 700 - 1500 Da[9]. Additionally, PL inhibitory activity of ***b4*** was increased 6 times respect to ***A3***.

3.2.3. Cholesterol esterase and pancreatic lipase inhibitory activities from RP-HPLC fractions

The fractions obtained by RP-HPLC process from ***B3*** were named: ***C1***, ***C2***, ***C3***, and ***C4*** (**Figure 5a and b**). These fractions were subjected to the assay of CE enzyme inhibition. On the other hand, the fractions obtained by RP-HPLC process from ***b4*** were named: ***c1*** and ***c2*** (**Figure 5c and d**). These fractions were subjected to the assay of PL enzyme inhibition.

Regarding CE enzyme inhibition assay, only the fraction ***C4*** inhibited the enzyme at 0.2 mg protein/mL (15.5 ± 1.5%). It is noteworthy that this fraction had the highest retention time in the RP-HPLC process, which would indicate that it is the most hydrophobic fraction. In this regard, Mudgil *et al.*[19] reported that hydrophobic amino acids obtained from camel milk could be responsible for the inhibitory activities on CE.

Moreover, it was proposed that the presence of hydrophobic residues could be a necessary characteristic for CE inhibitory peptides.[20]

On the other hand, both fractions obtained from ***b4*** by RP-HPLC process (***c1*** and ***c2***) inhibited the PL enzyme at 0.06 mg protein/mL. However, fraction c1 showed higher inhibitory activity than ***c2*** ($p < 0.05$), values being 51.9 ± 2.3 and 19.1 ± 3.3 % for ***c1*** and ***c2***, respectively. The values obtained in this work are similar than those obtained by Prados *et al.*[9] In this regard, they reported a 55% inhibition value for the most active peptide fraction evaluated at 0.04 mg proteins/mL. Note that PL inhibitory activity in ***c1*** was concentrated 15 times respect to the starting fraction obtained by FPLC (***b4***).

3.3. Identification of peptides using MALDI-TOF tandem mass spectrometry

In order to characterize the molecular mass and amino acid sequence of the peptides from the fractions ***C4*** and ***c1***, analysis by MALDI-TOF tandem mass spectrometry was performed.

A search for MS/MS fragments in MASCOT allowed identifying three peptides (**Figure 6**). For the fraction ***C4*** (highest CE inhibition) it was possible to identify a peptide, whose sequence was WNIHMEHQDLTTME (mass/load (m/z): 1802.36, +1). In the case of ***c1*** (highest PL inhibition), two peptides were identified: DFGIASF (m/z: 755.99, +1) and LAAVEALSTNG (m/z: 1044.95, +1). The three peptides identified were statistically significant ($p<0.05$), thereby confirming their identity in a 95% confidence.

As mentioned above, the most active fraction against CE obtained from the FPLC had a MW higher than 8000 Da (**Figure 3a**). However, the peptide identified from ***C4*** after the RP-HPLC process and MS/MS analysis showed a smaller size (1802.36 Da). This

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may be due to hydrophobic interactions between the peptides, which generate large aggregates that are not resolved in gel filtration chromatography. Note that this chromatographic process was carried out in native conditions. In line with this, Prados *et al*.[9] found that the most active fraction against CE obtained from 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 MS/MS analysis.

Regarding the sequence of peptide identified for CE, it was observed a high proportion of hydrophobic amino acid (42%). In line with this, this peptide was the most hydrophobic among identified peptides (**Table 1**). As mentioned before, it has been proposed that the presence of hydrophobic residues could be a necessary characteristic for CE inhibitory peptides.[20] An additional characteristic observed in CE inhibitor peptides is the presence of basic amino acids in the sequence.[17] In this sense, 14% of the amino acid residues in this peptide correspond to His (cationic amino acid), which were not present in PL inhibitory peptides.

In the case of PL inhibitory peptides, the two identified peptides had a molecular size within the range expected. Note that the most active fraction against PL obtained from FPLC process had a MW around 1000 Da. As mentioned before, the peptides reported to date with the ability to inhibit PL enzyme showed molecular size between 700 - 1500 Da.[9]

Regarding the sequence of PL inhibitory peptides, the two identified peptides showed high proportion of hydrophobic amino acid residues such as: Phe, Ile, Ala, Val, and Gly. This is consistent with the results obtained from the amino acid profile (**Figure 2**). In addition, it matches the high retention times obtained in the separation by RP-HPLC process (**Figure 5c and d**). Moreover, the hydrophobicity obtained from *in silico* study

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for these peptides was highly positive (**Table 1**). Regarding these values, they were higher than the hydrophobicity reported for hydrophobic proteins, like casein, meat, and wheat gluten (1.14, 1.06, and 0.97 kcal/mol, respectively).[21]

Interestingly, the three peptides identified also showed acidic amino acid residues (Asp and Glu), and/or their amines (Asn and Gln). These amino acids were reported in peptides with lipid-lowering activity.[9,22] Thus, a common factor in this type of bioactive peptides could be the presence of hydrophobic and polar amino acids, constituting amphipathic peptides capable to integrate lipid micelles in the intestinal lumen, or interact with micelle surface interfering with CE and PL enzyme activities.

3.4. *In silico* gastrointestinal digestion of peptides

Hypolipidemic peptides such as CE and PL inhibitors must be able to survive the gastrointestinal environment to exert its biological action at local level. Therefore, it is important to evaluate their stability or their bio-accessibility using systems where the effect of gastrointestinal digestion is considered. In this sense, an *in silico* simulation of gastrointestinal digestion was performed, using the hydrolysis tool of the BIOPEP-UWM program. The results obtained indicated that the three peptides were susceptible to enzymatic hydrolysis (**Table 1**). Therefore, if these peptides are not protected from the gastrointestinal environment, they will be hydrolyzed by digestive proteases, and their bioactivity modified.[23] In this regard, these peptides could be a source of new ones with greater, equal or lesser activity. If the latter were the case, its encapsulation should be evaluated to preserve the lipid-lowering activities in the gastrointestinal environment.

3.5. Molecular docking

Cholesterol esterase (E.C. 3.1.1.13) is a serine hydrolase which utilize the catalytic triad (Ser194, Asp320, and His435) residues for its mechanism. The catalytic domain of CE

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contains large and small domains and the active site of this enzyme is present almost at the center of these two domains.[24] On the other hand, pancreatic lipase (E.C. 3.1.1.3) has a catalytic triad (Ser152, Asp176, and His263) in the N-terminal domain, which access is controlled by a surface loop, the lid.[25]

To shed light on enzyme-peptides binding interactions, *in silico* molecular docking was performed in a first stage using the CABS-dock server. Starting from crystalline structure of CE or PL enzyme and amino acid sequence of putative ligands, this server perform docking search for the binding site allowing for full flexibility of the peptide and small fluctuations of the receptor backbone.[26] Interaction scheme of the model with the lowest average RMSD value, and contact protein and peptide residues at a cut off distance of 4.5 Å are showed in **Figure 7**. It can be seen firstly that CE-WNIHMEHQDLTTME and PL-LAAVEALSTNG docking performed showed a high quality prediction, average RMSD values being lower than 3 Å.[26] For PL-DFGIASF, a medium quality prediction was obtained (RMSD values between 3 to 5.5 Å).[26] Moreover, none of the three peptides interacted with the active site of the enzyme. In this sense, the three peptides from the BSG hydrolyzate that inhibit CE or PL would act by a non-competitive inhibition mechanism, where the inhibitor binding site is different from the substrate binding site. Additionally, the two PL inhibitory peptides bound at the same enzyme site, which could indicate a common binding site for this type of bioactive compounds.

In a second stage, a high-resolution structural and energy refinement of the peptide in the binding site was performed using FlexPepDock server, and interface energies were extracted. These energies values do not have a direct conversion to physical energy units like kcal/mol.[27] The values obtained were -22.35 ± 0.18, -10.46 ± 0.51, and -12.55

± 0.27. These negative values confirm that the interaction of the evaluated ligands with CE or PL enzymes was possible and favorable.

4. Conclusions

Fractions of peptides with cholesterol esterase and pancreatic lipase inhibitory activities were identified from BSG hydrolysate after a fractionation and purification process using different types of chromatography. Each chromatographic technique used allowed concentrating the evaluated bioactivity. Moreover, this is the first report which identified cholesterol esterase and pancreatic lipase inhibitory peptides from BSG.

MALDI-TOF tandem mass spectrometry allowed identifying three bioactive peptides: WNIHMEHQDLTTME (with potential CE inhibitory activity), DFGIASF and LAAVEALSTNG (with potential PL inhibitory activity). The three peptides found showed hydrophobic and acidic amino acid residues (Asp and Glu), and/or their amines (Asn and Gln), which could be a common feature among lipid-lowering peptides. These amphipathic peptides could be capable to integrate lipid micelles in the intestinal lumen, or interact with micelle surface interfering with CE and PL enzyme activities. Moreover, the molecular docking established a favorable interaction between enzymes and peptides, and a probably non-competitive inhibition mechanism for all the peptides evaluated.

Although it was possible to obtain the sequence of three peptides with inhibitory potential of enzymes related with lipid metabolism, further studies, including its chemical synthesis and *in vivo* assays, should be carried out to confirm its bioactivity and mechanism of action.

406 This work showed that the production of BSG hydrolysate could provide added value to
407 the by-product obtained from the beer industry.

408 **Conflict of Interest**

409 There are no conflicts to declare.

410 **Acknowledgments**

411 AGG, REC and MEA carried out the experiment. AGG, REC and SRD analyzed the
412 data and wrote the paper, and had primary responsibility for final content. All authors
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Figure Captions

Figure 1. Inhibition of the cholesterol esterase (CE) (**a**) and pancreatic lipase (LP) (**b**) enzymes by the anionic exchange chromatography fractions (***A1-A4***) evaluated at 0.4 mg protein/mL. Bars with different letters indicate significant differences ($p < 0.05$).

Figure 2. Amino acid profile of the most bioactive fractions obtained by anionic exchange chromatography (***A1*** and ***A3***).

Figure 3. FPLC profile of fractions ***A1*** (**a**) and ***A3*** (**b**). The molecular sizes (Da) of the most representative peaks of the collected fractions are shown.

Figure 4. Inhibition of the cholesterol enzyme (CE) by the FPLC fractions obtained from ***A1*** at 0.4 mg protein/mL (**a**); inhibition of the pancreatic lipase enzyme (PL) by the FPLC fractions obtained from ***A3*** at 1.4 mg protein/mL (**b**). Bars with different letters indicate significant differences ($p < 0.05$).

Figure 5. RP-HPLC profile of the fraction ***B3*** at 280 nm (**a**) and at 220 nm (**b**). RP-HPLC profile of the fraction ***b4*** at 280 nm (**c**) and at 220 nm (**d**).

Figure 6. Fragmentation spectrum of the three peptides identified from the most bioactive fractions (***C4*** and ***c1***): cholesterol esterase inhibition assay (**a**) and pancreatic lipase inhibition assay (**b** and **c**).

Figure 7. Structure, average RMSD value (Å), and contact protein-peptide residues of the best model generated from CABS-dock results. The peptide evaluated is show in red colour.

Table 1. Load mass ratio (m/z), *in silico* hydrophobicity and *in silico* simulation of gastrointestinal digestion (GID) of identified peptides

Identified peptides	m/z (+1)*	Hydrophobicity (kcal/mol)*	GID**
WNIHMEHQDLTTME	1802.36	+19.78	WNI - HMEHQDL - T - T - ME
DFGIASF	755.99	+9.11	DF - G - I - A - S - F
LAAVEALSTNG	1044.95	+12.78	L - A - A - V - EA - L - S - T - NG

*Obtained with *PepDraw* program. **Obtained with BIOPE-UWM tools using trypsin, pepsin and pancreatin as enzymes.

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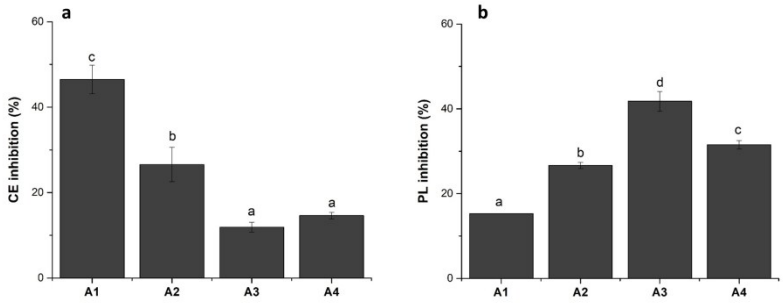

Figure 1. Inhibition of the cholesterol esterase (CE) (a) and pancreatic lipase (LP) (b) enzymes by the anionic exchange chromatography fractions (A1-A4) evaluated at 0.4 mg protein/mL. Bars with different letters indicate significant differences ($p < 0.05$).

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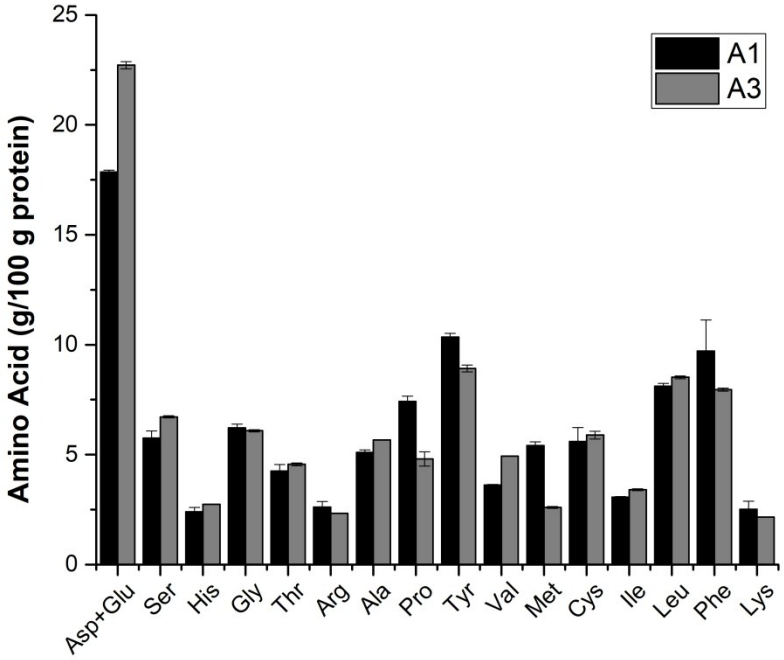

Figure 2. Amino acid profile of the most bioactive fractions obtained by anionic exchange chromatography (A1 and A3).

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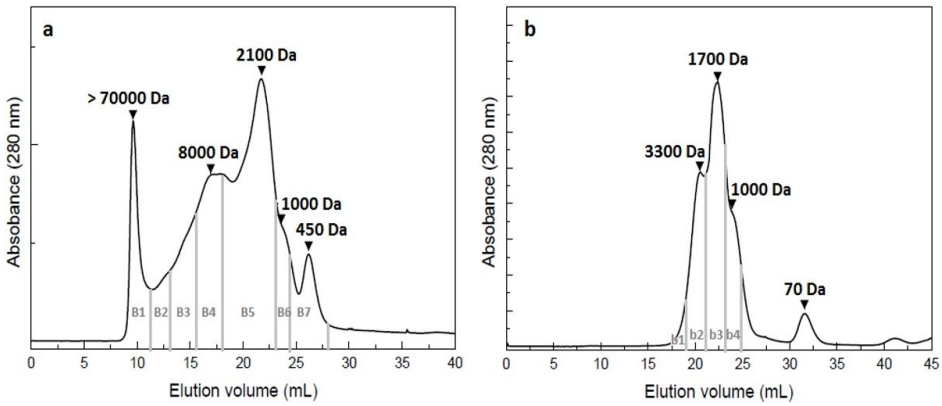

Figure 3. FPLC profile of fractions A1 (a) and A3 (b). The molecular sizes (Da) of the most representative peaks of the collected fractions are shown.

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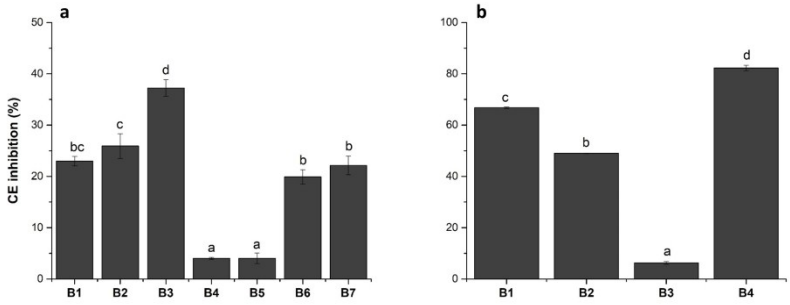

Figure 4. Inhibition of the cholesterol enzyme (CE) by the FPLC fractions obtained from A1 at 0.4 mg protein/mL (a); inhibition of the pancreatic lipase enzyme (PL) by the FPLC fractions obtained from A3 at 1.4 mg protein/mL (b). Bars with different letters indicate significant differences ($p < 0.05$).

278x99mm (300 x 300 DPI)

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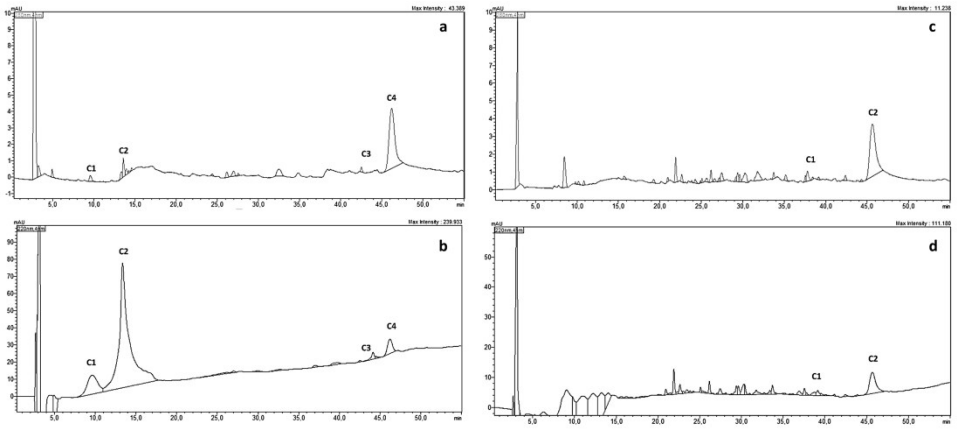

Figure 5. RP-HPLC profile of the fraction B3 at 280 nm (a) and at 220 nm (b). RP-HPLC profile of the fraction b4 at 280 nm (c) and at 220 nm (d).

338x190mm (300 x 300 DPI)

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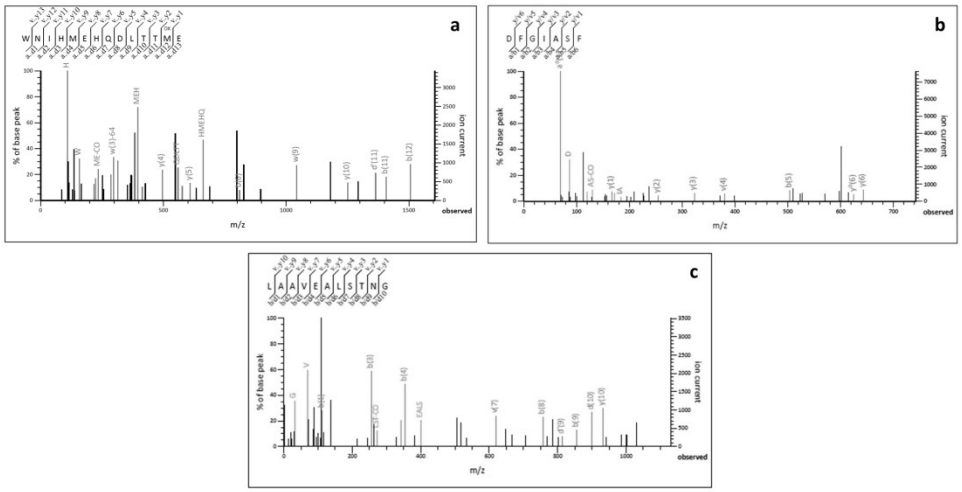

Figure 6. Fragmentation spectrum of the three peptides identified from the most bioactive fractions (C4 and c1): cholesterol esterase inhibition assay (a) and pancreatic lipase inhibition assay (b and c).

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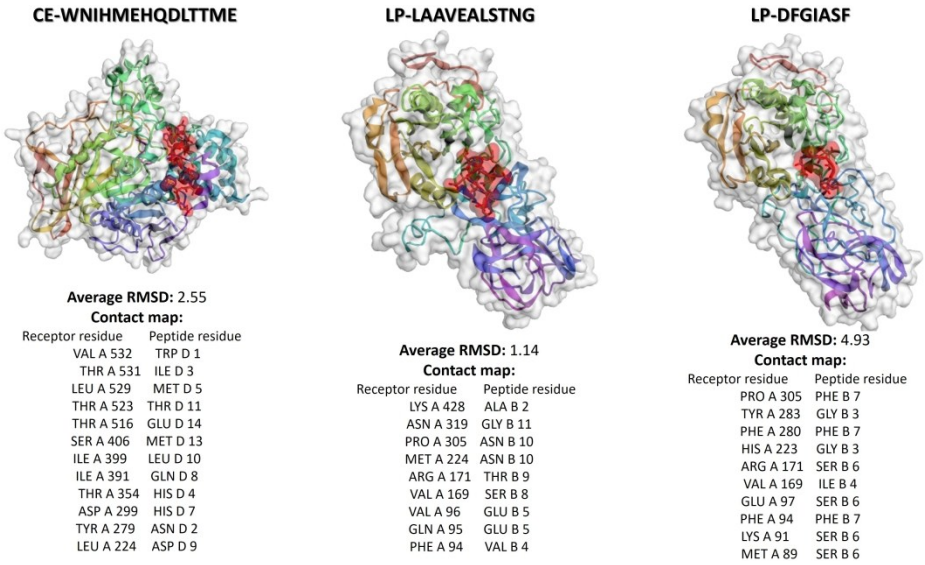

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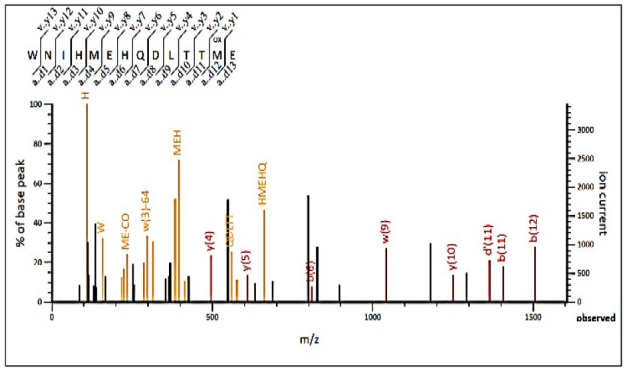
338x190mm (300 x 300 DPI)

Hydrolysis with proteases

Brewer´s spent grain hydrolysate

Lipid-lowering activity (*Cholesterol esterase* and *Pancreatic lipase* enzyme inhibition)

Isolation and identification of new lipid-lowering peptides



In silico analysis