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Sequential alcalase and flavourzyme treatment for preparation of α-amylase, α-glucosidase, and dipeptidyl peptidase (DPP)-IV inhibitory peptides from oat protein

Lourdes Ramírez Fuentes, Caroline Richard, Lingyun Chen *

Department of Agricultural, Food and Nutritional Science, 3-18 M Agriculture/Forestry Ctr, University of Alberta, 116 St. and 85 Ave, Edmonton, AB T6G 2P5, Canada

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ABSTRACT

The current study reported oat protein as a precursor for α-amylase, α-glucosidase, and dipeptidyl peptidase (DPP)-IV inhibitory peptides and studied the antidiabetic activities related to their structures. Enzyme inhibition assays *in vitro,* using oat protein treated by alcalase and flavourzyme fractionated into different molecular weights and hydrophobicity, indicated that the relatively hydrophobic fraction of 1–5 kDa inhibited enzymes related to glucose digestion, absorption, and metabolism activities. The α-amylase and DPP-IV were inhibited 57 and 78%, respectively, even at low peptide concentrations. LC-MS/MS analysis of the most effective fractions disclosed two eight amino acid sequences, identified from 12S oat globulin (GDVVALPA and DVVALPAG), and other sequences rich in amino acids like proline, leucine, valine, phenylalanine, and glutamine. The results suggest that proline and hydrophobic amino acids may favor hydrophobic interactions and hydrogen bonding with the target enzymes, especially the Leu-Pro sequence found in potent DPP-IV inhibitors.

1. Introduction

The increased prevalence of diabetes around the world represents a significant problem in public health. According to the International Diabetes Federation report (IDF Diabetes Atlas 9th edition, 2019), 90% of the worldwide population with diabetes have type 2 diabetes mellitus (T2DM). The affected population is prone to develop comorbidities, which means the presence of other chronic diseases such as high blood pressure, renal failure, retinopathy, or heart disease (Van Smoorenburg, Hertroijs, Dekkers, Elissen, & Melles, 2019). The diabetic population is generally under treatments that could require more than one medication to control glucose levels and treat any complications. Nowadays, great efforts are being taken in research to understand the role of food and nutrition in T2DM management since prevention and remission is feasible for some patients (Hopkins et al., 2020). Current management relies on dietary and lifestyle modifications and the use of anti-diabetogenic drugs such as insulin, biguanides, α-glucosidase inhibitors, dipeptidyl-peptidase (DPP)-IV inhibitors, incretin mimetics, meglitinides, among others (Olokoba, Obateru, & Olokoba, 2012). Despite their good antidiabetic effects, medications might be associated with side effects, which in the long term reduces the adherence to the treatment together with other factors like medication costs (García-Pérez et al., 2013).

Currently, the search for food sources to generate compounds with potential biological activities has become of great interest. Both animal and plant proteins are sources of the so-called bioactive peptides, defined as specific protein fragments that positively impact the functioning or conditions of living beings, thereby improving their health (Möller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008). The most studied antidiabetic activities from food derived peptides include α-amylase, α-glucosidase, and DPP-IV inhibitors. The first two are involved in polysaccharides' hydrolysis into monosaccharides to down-regulate blood glucose levels (Lebovitz, Irl Hirsch, & Vassello, 1997; Patil, Mandal, Tomar, & Anand, 2015). The main action of DPP-IV inhibitors is preventing the fast degradation of incretins like GLP-1 from producing a lasting effect on insulin stimulation (Ahrén, 2007). Thus, these enzymes are targets in developing peptides with antidiabetic activities from food proteins, which may provide an excellent opportunity for food and natural health products sectors to create diabetes-friendly foods to prevent and manage T2DM.

Oat is one of the most abundant cereals cultivated worldwide. Canada is known for being one of the largest producers of oats in its western prairie provinces (Yan, Fetch, Frégeau-Reid, Rossnagel, & Ames, 2011). Oat is a good source of both protein and dietary fibre. Oat β-glucan is

* Corresponding author.
E-mail address: lingyun.chen@ualberta.ca (L. Chen).

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known for its cholesterol lowering and glucose regulating effects. In addition, oat is the only cereal containing globulin protein, avenalin, as its major protein component; thus, it is more nutritious than most cereals with a greater proportion of essential amino acids like lysine, methionine, cysteine, and tryptophan (Peterson, 2011; Tiefenbacher, 2017). Therefore, oat is an interesting cereal to develop diabetic-friendly food products. Studies have shown the release of bioactive compounds from oat protein achieved by bacterial and fungal enzymatic hydrolysis, proving their successful antioxidant and ACE-I inhibition activities. However, few studies have considered investigating proteins from oats as a source of antidiabetic peptides. Preliminary experiments in our lab showed potential antidiabetic activities of oat protein hydrolysates. This positive effect has also been reported recently by Wang, Du, et al. (2018) and Wang, Zhang, et al. (2018) and some oat peptide sequences that can contribute to DPP-IV inhibition were identified. Research on antidiabetic properties of digested oat proteins has gained interest; nonetheless, more information regarding oat protein hydrolysis by different enzymes and their combinations is still required. The peptide fractionation processes need further elucidation and more peptide sequences that contribute to the antidiabetic effects are yet to be identified. Filling these knowledge gaps will allow rational design of oat protein hydrolysis and fractionation processing to prepare and concentrate peptides with desirable sequences to maximize intended physiological properties and health benefits. This research aimed to prepare oat protein hydrolysates by alcalase and flavourzyme treatment and fractionate them based on their molecular weight (M_W) and hydrophobicity characteristics to generate potential antidiabetic peptides. The collected fractions were evaluated for their antidiabetic effects, including α-amylase, α-glucosidase, and DPP-IV inhibitory activities. The fractions with the highest effects were characterized for the peptide structures and sequences by LC-MS/MS.

2. Materials and methods

2.1. Materials

Naked oat grains (*Avena nuda* L.) were provided by Wedge Farms Ltd., Manitoba, Canada. The oat protein was extracted by the established method in our laboratory (Nieto-Nieto, Wang, Ozimek, & Chen, 2014). The protein content was determined to be 76%, using a combustion nitrogen analyzer (Leco Corporation, St Joseph, MI, USA), and a factor of 5.83 was used for protein conversion. Protease from *Aspergillus Oryzae* (flavourzyme ≥ 500 U/g) and from *Bacillus licheniformis* (alcalase ≥ 5000 U/g), 3,5-DNSA, sodium hydroxide, potassium sodium tartrate tetrahydrate, α-amylase from porcine pancreas (Type VI-B, ≥5 units/mg), α-glucosidase from *Saccharomyces cerevisiae* (Type I, ≥10 units/mg) were obtained from Sigma Aldrich (St. Louis, MO, USA). DPP-IV inhibitor screening kit was obtained from Abcam (Abcam, ON, Canada). Other used chemicals were of analytical grade.

2.2. Preparation of oat protein hydrolysates

Oat protein hydrolysis was conducted according to the method reported by Orsini Delgado, Tironi, & Añón (2011) with modifications. First, oat protein dispersion (1% w/v) was prepared (pH 10) and stabilized at 37 °C for 1 h before adding alcalase (≤5000 U/g) in a ratio of 8 μL/100 mg protein powder. The reaction was carried out for 4 h with the mixture continuously stirred by a magnetic bar at 37 °C and adjusted to pH 10 every 10 min for the first hour by adding NaOH 0.1 M. After the hydrolysis treatment, the enzyme was inactivated by heating at 85 °C during 10 min. The suspension was left to cool down at room temperature and then freeze dried to obtain the powder samples of hydrolysates. Continuous hydrolysis by alcalase and flavourzyme involved an initial alcalase hydrolysis as described above, followed by flavourzyme treatment (5 μL/100 mg sample) at pH 7. The reaction mixture was left stirring for 2 h at 50 °C. Finally, the enzyme was inactivated at 85 °C for 15 min, followed by centrifugation at 12,000*g* for 20 min, and filtration of the supernatants. Both alcalase hydrolysate (AH) and alcalase-flavourzyme (AFH) filtered fractions were tested for enzymatic inhibition. M_W distribution of the hydrolysates samples was evaluated using size exclusion chromatography (Agilent 1200 series HPLC system equipped with a TSKgel G3000SW$_{XL}$ column (7.8 × 300 mm, Tosoh Corp., TO, Japan)).

2.3. Membrane filtration of oat protein hydrolysates

The protein sample hydrolyzed by alcalase-flavourzyme was passed through an ultra/diafiltration system equipped with Centramate Cassettes filtration system (T-series Omega, Pall Life Sciences, Mississauga, ON, Canada) using membranes with M_W cut off values of 5 and 1 kDa. The fractions with M_W distribution of 1–5 kDa and 1 kDa were collected, lyophilized, and stored at 4 °C.

2.4. Amino acid composition analysis

The amino acid composition was performed at the Alberta Proteomics and Mass Spectrometry Facility using the Waters AccQ-Tag system (Waters Corp., Milford, Mass.). Hydrolyzed samples were derivatized in borate buffer with Waters AccQ-Fluor reagent at 55 °C for 10 min. Chromatographic analysis of the derivatized amino acids was done on an Agilent 1200 series HPLC system (Agilent, Santa Clara, Calif.). Samples were separated by a Waters AccQ-Tag column (3.9 × 150 mm) at 37 °C with a three eluent gradient solvent system (AccQ-Tag eluent, ACN, and water) at a flow rate of 1.5 ml/min. and detected at 254 nm using an Agilent G1365D multiple wavelength detector. Asparagine and glutamine were hydrolyzed to their corresponding acids and were quantitated as such.

2.5. Reversed phase high performance liquid chromatography (RP-HPLC) fractionation

The ultra-filtration fraction with high activity in antidiabetic assays (M_W 1–5 kDa) was further fractionated based on its hydrophobicity using an Agilent 1200 series HPLC system with reversed-phase column (Zorbax SB-C18 column, 4.6 × 150 mm; 5 μm) with the following linear gradient composed of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in ACN): 5% solvent B for 5 min and 5–40% solvent B for 30 min, 40–90% B for 10 min, and finally 5 min at 90% B. Gradient elution was performed at a flow rate of 0.5 ml/min and 60 °C. Peaks were monitored at a UV wavelength of 280 nm and collected as four fractions. After collecting a suitable volume, samples were freeze dried and used to evaluate their antidiabetic properties using the same methods described in Section 2.6.

2.6. Antidiabetic properties

2.6.1. α-Amylase enzymatic assay

The α-amylase inhibitory effect was evaluated according to Awosika and Aluko (2019) with some modifications. The freeze dried oat protein hydrolysates or fraction samples were dissolved in a 20 mM sodium phosphate buffer (pH 6.9 with 6.7 mM sodium chloride) and then diluted to different concentrations. Then 100 μL aliquots were added into test tubes and incubated with 100 μL of α-amylase solution (1.125 U/mL) for 10 min at room temperature. Next, 100 μL of 1% starch dissolved in the same 20 mM sodium phosphate buffer were added. The reaction was carried out for 10 min, then 200 μL of dinitrosalicylic acid (DNSA) color reagent (96 mM 3,5-DNSA, 2 M sodium hydroxide and 5.3 M potassium sodium tartrate tetrahydrate) was added to the mixture. Test tubes were placed for 5 min in a boiling water bath to inactivate the enzyme. After cooling to room temperature, 3 ml of double distilled water was added to the solution for the final absorbance reading at 540 nm using a Jenway 6300 spectrophotometer (Cole-Parmer scientific

experts, Staffordshire, UK). Acarbose was used as the positive control. Absorbances were corrected with a blank sample, and the inhibition activity was calculated as:

% Inhibition = (Absorbance of the control − Absorbance of the sample/Absorbance of the control) × 100.

2.6.2. α-Glucosidase enzymatic assay

The α-glucosidase inhibition effect was assayed according to the method by Kwon, Vattem, & Shetty (2006) with slight modifications. The freeze dried oat protein hydrolysate or fraction samples were dissolved in 100 mM phosphate buffer (pH 6.9) at different concentrations. A volume of 50 μL sample was mixed with a 100 μL of α-glucosidase solution (0.3 U/mL) and incubated for 10 min in a 96 well plate at room temperature. Upon addition of the 50 μL *p*-nitrophenyl-α-D-glucopyranoside solution (5 mM, as substrate), the sample absorbances at 405 nm were recorded using a multi-mode microplate reader (SpectraMax M3; Molecular Devices, San Jose, CA, USA) for 20 min. The obtained absorbances were corrected using a blank sample and compared to the non inhibited reaction control. Acarbose was used as the positive control. The inhibitory activity was calculated as:

% Inhibition = (Absorbance of the control − Absorbance of the sample/Absorbance of the control) × 100.

2.6.3. DPP-IV enzymatic assay

This assay was assessed by a DPP-IV inhibitor screening kit (ab133081, Abcam, ON, Canada) with minimal modification. The freeze-dried oat protein hydrolysates or fraction samples were dissolved in the assay buffer provided in the kit and then diluted to different concentrations (from 100 to 500 μg/mL). The DPP-IV enzyme was diluted at a 1:4 ratio (v/v) by the same assay buffer. The initial activity wells included 40 μL of assay buffer, 10 μL diluted DPP-IV, and 50 μL substrate. Inhibitory activity wells included 10 μL of oat protein hydrolysates or fraction samples, or sitagliptin (positive control), 30 μL of assay buffer, 10 μL of DPP-IV, and 50 μL of the substrate. The plate was incubated for 10 min at room temperature before starting the reaction by adding the substrate. Then, the plate was covered with a 96 well cover sheet and foil and incubated for 30 min at 37 °C. After incubation, the fluorescence readings were recorded using a multi-mode microplate reader (SpectraMax M3; Molecular Devices, San Jose, CA, USA) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The inhibitory activity was calculated as:

% Inhibition = (Initial activity − Inhibitory activity/Initial activity) × 100.

2.7. De novo peptide sequencing

The peptide fractions with the highest overall antidiabetic activities were subject to LC-MS/MS analysis on a q-Tof premier mass spectrometer (Waters, Milford, MA) coupled with a nano Acquity UPLC system (Waters, Milford, MA). 5 μL of the peptides were loaded onto a nano trap column (75 μm × 20 mm, Acclaim PepMapTM 100 nanoViper trap column, Thermo Fisher Scientific) followed by a nano analytical column (75 μm × 150 mm, Acclaim PepMapTM 100 nanoViper column, Thermo Fisher Scientific). Desalting on the peptide trap was achieved by flushing the trap with 1% solvent B (acetonitrile with 0.1% formic acid) and 99% solvent A (water with 0.1% formic acid) at a flow rate of 5 μL/min for 2–3 min. Solvents used were: Solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in acetonitrile at a flow rate of 350 μL/min with the following gradient elution: 1 to 75% solvent B for 40 min, 75–98% solvent B for 10 min, and 98% for 5 min. The mass spectrometer was operated at positive mode with a capillary voltage of 3.2 kV and a source temperature of 100 °C. Spectra were recorded over the (m/z) range of 175–813 Da in MS mode and 50–1990 Da in MS/MS mode. Instrumental control and data analysis were performed using MassLynx software (Micromass U.K. Ltd., Wythenshawe, Manchester, U.K.). De novo sequencing was done using Peaks X Pro (Bioinformatics Solutions Inc., Ontario, Canada).

2.8. Statistical analysis

Experiments were performed in triplicates, and results were presented as mean values ± standard deviation. IBM SPSS version 2.6 (International Business Machines, NY, USA) was used for the statistical analysis of the numerical data. Comparison of samples means was made through one-way analysis of variance (ANOVA) followed by Tukey pos-hoc test at a significance level of 0.05.

3. Results and discussion

3.1. Enzymatic hydrolysis of oat protein

Oat protein hydrolysis by enzymes like pepsin, trypsin, chymotrypsin was reported in previous *in silico* and *in vitro* analyses; however, some of these enzymes like pepsin showed a lower degree of hydrolysis alone, and other enzymes like trypsin cleaved large sequences of 7 to 25 amino acids long after hydrolysis (Cheung, Nakayama, Hsu, Samaranayaka, & Li-Chan, 2009; Vanvi & Tsopmo, 2016; Wang, Yu, Zhang, Zhang, & Fan, 2015b; Yu, Wang, Zhang, & Fan, 2016). Currently, the information on oat protein hydrolysis by other enzymes is scarce. It is known that alcalase is an endoprotease with a broad specificity in peptide cleavage and has been widely used to prepare bioactive peptides from food proteins (Tacias-Pascacio et al., 2020). Flavourzyme is a mixture of exo and endoproteases, known for its debittering capacity and generation of short chain peptides (Rubi, Campos, Peralta González, Guerrero, & Ancona, 2013; Sharma et al., 2019). Thus, alcalase and flavourzyme were selected in this work to achieve extensive hydrolysis of oat protein. The SE-HPLC chromatograms in Fig. 1 show oat protein's M_W distribution and hydrolysates by alcalase and alcalase-flavourzyme treatment. The major peaks in oat protein were in the range of 30–7.2 kDa, which progressively shifted to a more defined 6.1 kDa peak when hydrolyzed with alcalase. However, the obtention of smaller peptides was not efficient with the use of alcalase alone. Therefore, further hydrolysis was conducted with flavourzyme because it has been shown to produce smaller peptides due to its endo and exopeptidase action (Walters, Willmore, & Tsopmo, 2020). The peptides M_W was significantly reduced after flavourzyme hydrolysis with major peaks in the range of 5.7 kDa and 1.5 kDa because the pre-digestive effect of alcalase over internal peptide bonds of the protein structure favored flavourzyme cleavage of amino acids at the chain terminus. In previous literature, sequential alcalase and flavourzyme hydrolysis led to an increased degree of hydrolysis of chickpea protein of up to 50% and enhanced antioxidant activity of the obtained peptides (Xu et al., 2020). This, however, was the first time demonstrating that sequential alcalase and flavourzyme hydrolysis was effective in producing lower M_W polypeptide chains from oat protein.

3.2. Antidiabetic activities of oat hydrolysates.

3.2.1. α-Amylase inhibitory assay

Fig. 2A shows the α-amylase inhibitory effect of oat protein hydrolysates by alcalase treatment (AH) at different doses. With increasing hydrolysate concentration from 0.33 to 1.0 mg/mL, the inhibitory activity increased from 18.1 up to 49.5%. Further increasing AH concentration reduced the α-amylase inhibitory effect. Such trend was also observed by Powers & Whitaker (1978), where the presence of high concentrations of the inhibitor resulted in its dissociation from α-amylase or conformational changes that modified the inhibitor affinity to the enzyme. Another possible explanation could be attributed to the peptides' aggregation when increasing the concentrations instead of

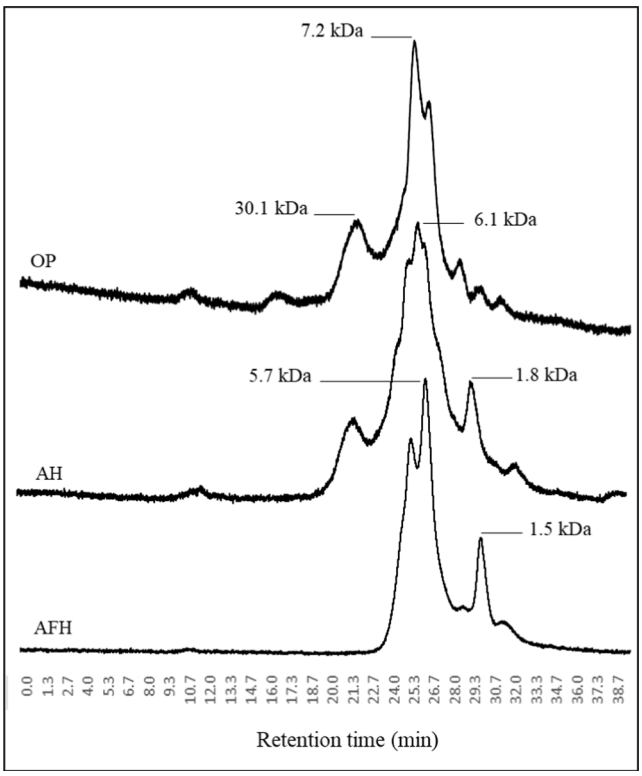

Fig. 1. Size exclusion chromatogram (UV wavelength 280 nm) of oat protein (OP) and oat protein alcalase (AH) and alcalase-flavourzyme (AFH) hydrolysates. M_W of oat protein and hydrolysates were obtained using a protein standard mix containing Ribonuclease A, Thyroglobulin, γ-Globulin, Albumin and *p*-aminobenzoic acid markers to calculate their log M_W and respective elution times ($R^2 = 0.98$).

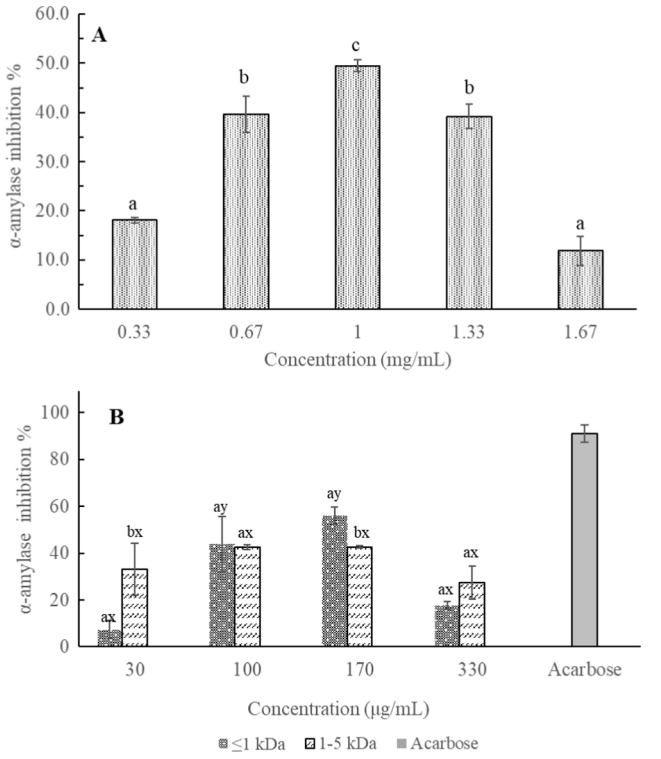

Fig. 2. (A) Inhibitory effect of α-amylase by alcalase hydrolysate (AH) at different concentrations. Different letters on top of the bars represent a significant difference between doses ($p < 0.05$). (B) Inhibitory effect of α-amylase by peptide fractions from alcalase-flavourzyme hydrolysate (AFH) at different concentrations compared to the control, acarbose at 30 μg/mL. Different letters (a–c) on top of the bars represent a significant difference between groups at the same concentrations ($p < 0.05$). Different letters (x–z) on top of the bars represent significant difference between the same fractions at various concentrations ($p < 0.05$).

binding to the enzyme for inhibition. Some of these peptides might be characterized by a net charge creating electrostatic interactions between oppositely charged peptides in addition to increased hydrophobic interactions when concentration increases (Dickinson & Leser, 2007). Inhibitory effect of AH from oat protein was higher than the inhibition activity reported for seaweed alcalase hydrolysates (~30%) at 1.86 mg/mL (Admassu, Gasmalla, Yang, & Zhao, 2018)

Fig. 2B shows the α-amylase inhibitory effect of oat peptide fractions from the alcalase-flavourzyme hydrolysate (AFH) with the M_W of 1–5 kDa and 1 kDa. Similar inhibitions of up to 42.6 ± 0.5% and 56.0 ± 3.7% were achieved for both fractions, respectively, at a concentration almost six times lower than the one required from AH. The 1–5 kDa fraction showed ~33% α-amylase inhibition even at a low concentration of 30 μg/ml, but further increasing the fraction concentration from 100 to170 μg/ml did not significantly improve the α-amylase inhibition. For the ≤1 kDa fraction, the α-amylase inhibition effect increased from ~7% to 44%, and 56% when the concentration increased from 30 to 100 and 170 μg/ml. Further increasing peptide concentration to 330 μg/ml reduced the inhibitory activity for both 1–5 kDa and ≤1 kDa fractions. The dose dependency effect was not clear for α-amylase inhibitory effect in this study, which is worthy of investigation in the future. Nonetheless, the trend indicates that the obtention of smaller peptides by combined alcalase and flavourzyme hydrolysis, followed by the hydrolysate filtration to recover the low M_W fraction, effectively concentrated the peptides with α-amylase inhibitory capacity. Similar results were obtained for pea protein alcalase hydrolysates where ultrafiltration of the sample into a fraction of 1–3 kDa increased the inhibitory effect (Awosika & Aluko, 2019). It is noticed that the oat peptide fraction (AFH) exhibited a similar or higher α-amylase inhibitory effect when compared to pea protein hydrolysate fractions. For example, AFH of 1–5 kDa showed an α-amylase inhibitory effect of 42.6 ± 1.1% at 100 μg/mL. To reach a similar level of α-amylase inhibitory, about 225 μg/mL was required for pea protein hydrolysate fraction of 1–3 kDa (Awosika & Aluko, 2019). In accordance with our study, the low M_W fraction of Pinto bean protein hydrolysates by ultrafiltration showed a higher α-amylase inhibitory effect of 62% at 500 μg/mL. (Ngoh & Gan, 2016). Although a similar molecular weight cut off was used in chia protein hydrolysate fraction with M_W of 1–3 kDa showed the inhibitory effect of 18% at a concentration of 10 mg/mL (Sosa Crespo, Laviada Molina, Chel-Guerrero, Ortiz-Andrade, & Betancur-Ancona, 2018). It should be mentioned that α-amylase inhibitory effects have been mainly observed for plant extracts and other non proteinaceous compounds (Bashary et al., 2019; de Sales, de Souza, Simeoni, Magalhães, & Silveira, 2012). The above comparison allows us to confirm that fractions from oat protein hydrolysates can potentially generate α-amylase inhibitory peptides.

These findings are consistent with those reported in other studies and support the idea that low M_W peptides have greater bioactivity as more active side chains on amino acid residues can be exposed outside to increase the possibilities of interaction with α-amylase in its catalytic site or subsites (Admassu et al., 2018; Ngoh & Gan, 2016; Oseguera-Toledo, Gonzalez de Mejia, & Amaya-Llano, 2015). The positive control, acarbose (82.9 ± 1.65 to 98.7 ± 1.0%), displayed a significantly higher α-amylase inhibitory effect even at a low concentration of 30 μg/mL *($p < 0.05$)*, which is expected for a well known synthetized antidiabetic drug. Oat peptides of a low M_W fraction achieved 30% of the inhibition at 30 μg/mL. The obtained inhibitory activity is still not comparable to acarbose; however, oat peptides present potential inhibitory activity that could be the base to generating a more natural

source of antidiabetic ingredient to delay α-amylase digestion of starch because peptides are natural with lower side effects. In addition, the use of bioactive peptides from food proteins may provide other biological functions as antihypertensives, antioxidants, and bactericides (Kannan, Hettiarachchy, & Marshall, 2011), among others. However, further research is needed to address health benefits and other stability concerns for food applications. Moreover, the peptides can be added in food formulation in larger amounts than drugs to exert health benefits.

3.2.2. α-Glucosidase inhibitory assay

In this study, the oat AH showed a significant difference in the inhibition percentage over time. The enzymatic assay showed α-glucosidase inhibitory effects of up to 38% in the first minute; however, the inhibitory effect significantly dropped by half after 5 min and then decreased to less than 10% after 10 min. The results obtained in this assay suggests that oat peptides presented a low affinity to the enzyme as the inhibitory effect was not observed for a long time. In this study, no concentration dependent effect was observed for oat AH. This could be due to the presence of a diversity of peptides with a wide range of molecular weights that could interfere with the inhibition of α-glucosidase. Higher concentrations of peptides may need to be tested in the future because other protein sources like hemp seeds alcalase hydrolysates showed inhibitory effects of around 58% and 25% at concentrations of 100 mg/mL and 100 μg/mL, respectively (Ren et al., 2016).

Inhibition of α-glucosidase has been observed for peptides from a greater variety of sources. For instance, the inhibitory effect was obtained for chia seeds by alcalase-flavourzyme treatment, where ~40% inhibition was obtained for fractions >10 kDa at 0.68 mg/mL; the 1–3 kDa and <1 kDa fractions showed inhibitory effects of 18 and 8%, respectively. In contrast, chia pepsin-pancreatin hydrolysates showed no inhibitory effect for both 1–3 and <1 kDa M_w fractions, whereas 80% inhibitory effect was obtained by the 5–10 and >10 kDa fractions (Sosa Crespo et al., 2018). These results imply that the inhibition of α-glucosidase relies on the specificity of enzymes for protein hydrolysis, as this will determine the cleaving sequence, the peptide's size, and its bioactivity (Awosika & Aluko, 2019). The α-glucosidase inhibitory effect was also observed for alcalase hydrolysates from yellow field pea and walnut (~38%), but at a higher concentration of 20 mg/mL, and the inhibition increased in a dose-dependent mode (Awosika & Aluko, 2019; Wang, Du, et al., 2018). One recent research also reported that an oat globulin derived peptide of 8 amino acids could inhibit α-glucosidase at the IC_{50} value of 78.58 μg/mL (Wang, Zhang, et al., 2018). These results suggest that as a plant protein-based source, oat protein can generate peptides as α-glucosidase inhibitors. It was also noticed that oat AFH fractions of ≤1 kDa and 1–5 kDa showed no obvious inhibitory effect (data not shown). These findings lead to the assumption that AFH in our research might be less efficient to generate peptides with a strong α-glucosidase inhibition effect compared to trypsin hydrolysates.

3.2.3. DPP-IV inhibitory assay

Oat AH tended to a dose dependent DPP-IV inhibitory effect (Fig. 3A) starting at 4.9 ± 1.5% and increasing to 48.7 ± 13% when increasing concentration from 100 to 500 μg/mL. However, the high inter sample variability prevented from reaching statistical differences. Nevertheless, a dose dependent inhibition was observed for the 1–5 kDa AFH fraction (Fig. 3B), which showed inhibition of 56.2 ± 4.3% when the peptide concentration increased to 500 μg/mL *($p < 0.05$)*. It has been reported studies on casein, soy and common bean proteins that peptides of low M_W had higher DPP-IV inhibitory effect, some of them with M_W of 10 kDa or 1 kDa (González-Montoya, Hernández-Ledesma, Mora-Escobedo, & Martínez-Villaluenga, 2018; Nongonierma & Fitzgerald, 2013; Oseguera Toledo, Gonzalez de Mejia, Sivaguru, & Amaya-Llano, 2016). In line with the previous research, ultrafiltration of protein hydrolysates provided an effective approach for concentrating peptides with DPP-IV inhibition effect.

In previous work by Wang, Yu, Zhang, Zhang, and Fan (2015a, 2015b), hydrolysates of oat globulin showed good DPP-IV inhibition (IC_{50} 2.04 mg/mL) after 14 h of trypsin treatment. In our study, the continuous hydrolysis by alcalase and flavourzyme was more efficient to release bioactive peptides with strong DPP-IV inhibition capacity (IC_{50} of 0.413 ± 15 mg/mL) in only 6 h. It is likely related to the fact that alcalase belongs to the endoprotease classification and flavourzyme exhibits both endo and exoprotease activity with non specific cleavage; thus, such combination allowed extensive hydrolysis of oat protein for more rapid generation of DPP-IV inhibitory peptides. Alcalase and flavourzyme continuous hydrolysis in chickpea protein demonstrated that this combination of enzymes is an effective treatment for plant proteins to increase cleaving sites to promote extensive hydrolysis and, therefore, improve the peptide's bioactivities (Xu et al., 2020). Porcine skin gelatin hydrolysates by alcalase and flavourzyme treatment demonstrated a DPP-IV inhibitory effect of approximately 60% at a concentration of 5 mg/mL (Huang, Hung, Jao, Tung, & Hsu, 2014). In comparison, oat protein hydrolysates presented a similar activity at a lower concentration of 500 μg/mL. Velarde-Salcedo et al. (2013) showed that hydrolysates from amaranth, black bean, soybean, and wheat by enzymes (pepsin, trypsin, and pancreatin) in simulated gastrointestinal digestion had a DPP-IV inhibitory effect of 20 to 60% at the concentration of 1.4 mg/mL. Moreover, AFH fractions from oat protein were comparable to hydrolysates from lactoferrin and bovine serum albumin that displayed IC_{50} values of 0.379 and 0.513 mg/mL, respectively (Lacroix, Chen, Kitts, & Li-Chan, 2017). As expected, DPP-IV positive control inhibitor, sitagliptin, had the highest inhibitory effect (86.8% at a much smaller concentration of 50 μg/mL). -Although oat hydrolysates did not reach as high inhibitory effects as the positive control, findings in this study confirm that DPP-IV inhibitory peptides from oat protein are comparable to other peptides from various plant and animal protein sources. It would also be necessary to study the inhibitory effect at higher concentrations in the future to understand the peptide's inhibitory dose

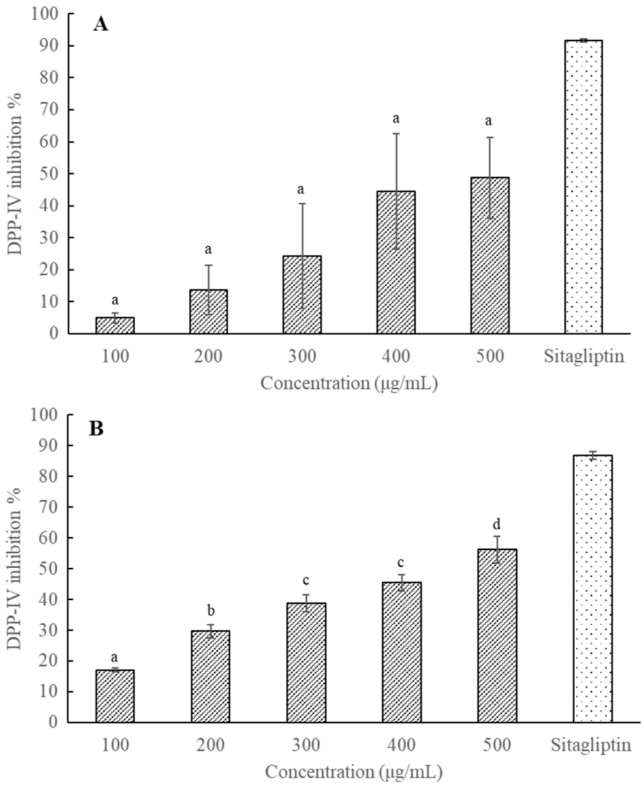

Fig. 3. Oat alcalase hydrolysate (AH) (A) and alcalase-flavourzyme hydrolysate (AFH) 1–5 kDa fraction (B) concentration dependent inhibitory effect of DPP-IV. Different letters on top of the bars represent significant difference between concentrations ($p < 0.05$).

dependent effect.

3.3. RP-HPLC peptide fractionation

Since the AFH fraction of 1–5 kDa showed a weak inhibitory effect in α-glucosidase and medium inhibitory activities in α-amylase and DPP-IV assays, it was chosen for further fractionation using RP-HPLC. In this study, oat AFH was separated into four fractions (F1, F2, F3, and F4), as shown in Fig. 4, with F1 being the most hydrophilic and F4 the most hydrophobic fraction. All four fractions were then collected and tested for their capacity to inhibit α-amylase, α-glucosidase, and DPP-IV.

Specific fractions exhibited better inhibitory effects. For instance, a stronger α-amylase inhibition effect was observed for F2 and F3, as shown in Fig. 5A. Specifically, the α-amylase inhibitory effect was 53.8 ± 5.6% for F2 even at a low concentration of 30 μg/mL when compared to 33% for 1–5 kDa fraction at the same concentration. It was noticed that the inhibitory effect decreased with increasing concentration of the F2 to 170 μg/mL. For F3, concentrations of 30 and 100 μg/mL generated the strongest α-amylase inhibitory effects of 46.6 ± 6.3 and 57.3 ± 9.5%, respectively. The α-amylase inhibitory effect observed for F2 and F3 (F1 and F4 effect not shown as they showed none or very weak inhibitory effects) suggests that peptides with medium polarity are necessary for interaction with the active site of α-amylase (González-Montoya et al., 2018). Studies where RP-HPLC is used to fractionate peptides with α-amylase inhibitory activity are lacking. However, limited works that analyzed the amino acid sequences of peptides have shown that both polar and non-polar amino acids are required in the peptide sequences to bind the active site of α-amylase for its inhibition (González-Montoya et al., 2018; Jhong, Riyaphan, Lin, Chia, & Weng, 2015; Yu, Yin, Zhao, Liu, & Chen, 2012).

For the α-glucosidase assay, the inhibition values at 10 min were used to compare the effect of four fractions, as 10 min was the longest time that oat peptides generated an inhibitory activity. The results in Fig. 5B showed the α-glucosidase inhibitory effect for F1 and F2 since F3, and F4 show little or no inhibitory effect. The inhibitory effect of F1 at a low concentration of 25 μg/mL was 19.7%, which decreased with increasing concentration to 75 and 125 μg/mL. The inhibitory effect of F2 was 33.4 ± 1.8% at the highest concentration of 125 μg/mL, suggesting that the oat peptide fractions with medium polarity and the possible presence of hydrophilic amino acids might better inhibit α-glucosidase. However, studies report that the hydrophobicity of peptides and α-glucosidase inhibitory effects were poorly correlated (Ibrahim, Bester, Neitz, & Gaspar, 2018). Similar α-glucosidase inhibitory effects that ranged from 27 to 33% at 1000 μg/mL were found for peptide fractions generated from germinated soy protein hydrolysate (González-Montoya et al., 2018). As a positive control, acarbose showed an α-glucosidase inhibitory effect of 37.1% at a concentration of 125 μg/mL, which is in accordance with findings reported in the literature (Lordan, Smyth, Soler-Vila, Stanton, & Paul Ross, 2013; Moon et al., 2011). The low inhibitory effects of both oat peptides and the positive control could be due to the structural difference that comes with α-glucosidase's origin or source (Gao & Kawabata, 2014; Lacroix et al., 2017). Such difference was discussed in previous studies where polyphenols or protein hydrolysates from tea, yogurt, fish sauce, plant extracts, and chicken essence showed inhibitory activity towards yeast α-glucosidase, while no inhibition towards α-glucosidase from rat small intestine. On the other hand, green and oolong tea presented inhibition activity for both types of glucosidases (Matsui, Oki, & Osajima, 1999; Shai, Magano, Lebelo, & Mogale, 2011). Acarbose is considered the best rat intestinal α-glucosidase inhibitor but not as effective when inhibiting yeast α-glucosidase (Kim et al., 1999). These findings suggest that different factors can impact enzymatic inhibition, one of which is the studied enzyme's origin or source. Research on the second type of α-glucosidase is required to understand better the potential antidiabetic response of the oat AFH fractions in the future.

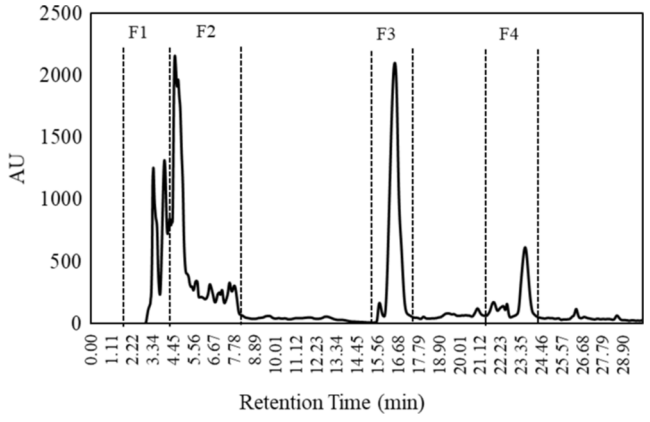

Fig. 4. Reverse phase chromatogram of four fractions obtained from AFH sample of 1–5 kDa.

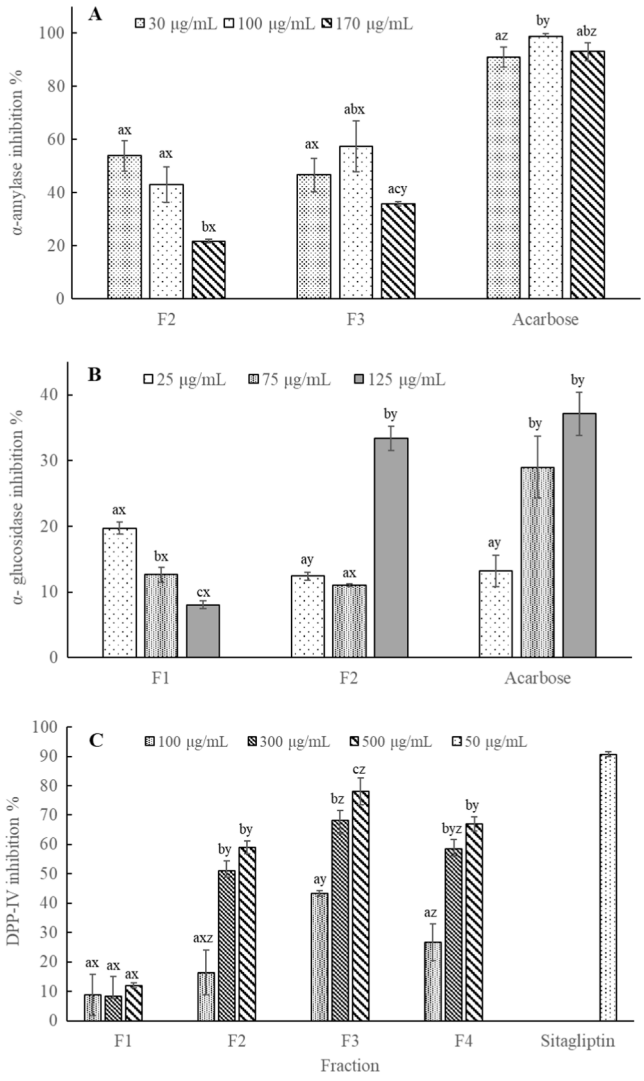

Fig. 5. α-Amylase inhibition by fraction 2 (F2), fraction 3 (F3), and the control, acarbose (A); α-glucosidase inhibition by fraction 1 (F1), fraction 2 (F2), and acarbose after 10 min incubation (B); DPP-IV inhibitory effect by fraction 1 (F1), fraction 2 (F2), fraction 3 (F3), fraction 4 (F4), and the control, sitagliptin (C). Different letters (a-c) on top of the bars represent significant difference between concentrations of the same fraction ($p < 0.05$). Different letters (x-z) on top of the bars represent a significant difference between fractions at the same concentrations ($p < 0.05$).

F1, F2, F3, and F4 all exhibited DPP-IV inhibitory effects. Especially, F3 showed an inhibitory effect of 43.3% at a relatively low concentration of 100 ug/mL (Fig. 5C). Further increasing concentration to 500 μg/mL led to an increased inhibitory effect of 78.0%. The higher inhibitory effect observed for F3 and F4 suggests that hydrophobic amino acids strongly contribute to peptides' DPP-IV inhibition. The existence of some hydrophilic amino acids might further improve the peptide bioactivity as F3 shows the highest inhibitory effect. Nongonierma & FitzGerald (2019) also reported that the hydrophobic or aromatic amino acids in peptide sequences contributed to DPP-IV inhibition. Moreover, structural analysis of known inhibitors of DPP-IV suggests their capacity to bind to the active site of the enzyme by hydrogen bonding and hydrophobic interactions (Chakraborty, Hsu, & Agoramoorthy, 2014). Thus, it is believed that the exposure of hydrophobic and aromatic amino acids, obtained by the hydrolysate of low M_W (1–5 kDa), contributed to the strong capacity of oat peptides to inhibit DPP-IV and the fractionation by RP-HPLC to further concentrate the peptides with DPP-IV inhibitory effects. It should be mentioned that the highest DPP-IV values reached ~50% for oat protein hydrolysate fractions in this study. It would be necessary to study the inhibitory effect at higher concentrations in the future to understand the peptide's inhibition dose dependent effect.

3.4. Amino acid content and peptide sequencing of the effective fractions

The amino acid compositions of oat protein and the fractions from its AHF hydrolysates are shown in Table 1. Glx (Glu + Gln) and Asx (Asp + Asn) decreased significantly after hydrolysis, and this is consistent with the fact that both acidic and basic subunits of oat globulin are susceptible to endo and exopeptidases since these oat polypeptides are rich in Glu and Asp, respectively (Burgess, Shewry, Matlashewski, Altosaar, & Miflin, 1983; Liu et al., 2009; Nieto-Nieto et al., 2014). Another study demonstrated that flavourzyme caused an increased exposure of aromatic amino acids, which is in line with the observed increment in Phe and Tyr in the AFH samples in our study (Walters et al., 2020). Alcalase, on the other hand, has preferential cleavage before and after hydrophobic amino acids (Esfandi, Willmore, & Tsopmo, 2018), which explains the significant increase in hydrophobic amino acids (e.g. Val, Ile, Leu, Phe) in the AFH fractions (53.9% and 47.5%), compared to the original oat protein (41.5%) *($p < 0.05$)*. The increased aromatic and hydrophobic amino acids provide a possible explanation of the improved inhibitory effects after enzymatic hydrolysis, together with reduced M_W to expose active groups.

For obtention of α-amylase inhibitory effect, it is considered important for the peptide chain to contain a Pro residue at the N- or C-terminus or both, as well as Gly or Phe at the N-terminus and Phe or Leu at the C-terminus (Ngoh & Gan, 2016). De novo sequencing of our sample also identified new sequences (Table 2) varying from 4 to 7 amino acid peptides with similar amino acid content and position characteristics. For example, Phe-Pro-Leu-Leu-Gln (FPLLQ), Phe-Pro-Leu-Leu-Phe (FPLLF), and Phe-Pro-Leu-Leu-Leu (FPLLL) all have Phe at the N-terminus and the presence of Leu and Phe at the C-terminus. Moreover, two peptide sequences of 8 amino acids each were identified from F3: Gly-Asp-Val-Val-Ala-Leu-Pro-Ala (GDVVALPA) and Asp-Val-Val-Ala-Leu-Pro-Ala-Gly (DVVALPAG). Both peptides are constituted by hydrophobic amino acids and one negatively charged amino acid (Asp). The high amount of Val, Ala, and Leu observed in the amino acid composition of samples (Table 1) matches their appearing frequency in the identified sequences. Reported sequences from seaweed Gly-Gly-Ser-Lys (GGSK) and Glu-Leu-Ser (ELS) exhibited a non competitive type of inhibition when binding to the allosteric site of α-amylase, thus, creating conformational changes in the enzyme's structure and modifying the affinity to its substrate (Arise, 2016; Powers & Whitaker, 1978). Similar amino acids were found in the peptide sequence Glu_{pyro}-Val-Phe-Gly-Lys (E_{pyro}VFGK) from F3; however, mechanisms of action are yet to be investigated.

For the α-glucosidase assay, molecular docking studies suggest that an albumin peptide Lys-Leu-Pro-Gly-Phe (KLPGF) is comparable to acarbose as a positive control for α-glucosidase inhibition with similar IC_{50} values of 33 and 39 mg/mL, respectively (Yu et al., 2012). A similar pattern was observed in the effective sequences identified in this study. For example, Leu-Pro-Pro-Gln-Leu (LPPQL), Phe-Pro-Leu-Leu-Gln (FPLLQ), and Leu-Pro-Glu-Leu-Gln (LPELQ) (Table 2) both contain Leu-Pro or Pro-Leu as found in other peptides registered as α-glucosidase inhibitors (Minkiewicz, Iwaniak, & Darewicz, 2019). It was noticed that F2 from oat protein exhibited a fair degree of inhibition compared to acarbose (IC_{50} 137.7 ± 23.2 μg/mL). According to an analysis of structural properties, α-glucosidase inhibition seems to rely more on the presence of hydrogen bonding. Thus, amino acids such as Ser, Thr, Tyr, Lys, or Arg at the N-terminus and/or a Pro at the penultimate position of

Table 1
Amino acid composition of oat protein and the fractions from its AHF hydrolysates.

Residue	Oat Protein		<1 kDa Fraction		1–5 kDa Fraction	
asx*	8.47 ±	0.09_a	5.86 ±	0.04_b	6.21 ±	0.01_c
ser	5.86 ±	0.04_{ab}	5.68 ±	0.08_a	5.93 ±	0.01_b
glx*	21.97 ±	0.09_a	13.48 ±	0.11_b	15.40 ±	0.09_c
gly	7.52 ±	0.08_a	7.46 ±	0.09_a	7.40 ±	0.05_a
his	2.19 ±	0.03_a	3.03 ±	0.04_b	2.81 ±	0.02_c
arg	6.39 ±	0.16_a	3.24 ±	0.07_b	4.73 ±	0.02_c
thr	4.01 ±	0.01_a	4.38 ±	0.06_b	4.25 ±	0.03_b
ala	6.90 ±	0.01_a	8.20 ±	0.10_b	7.01 ±	0.13_a
pro	6.59 ±	0.14_a	3.72 ±	0.09_b	4.86 ±	0.03_c
cys	2.61 ±	0.07_a	n.d.		1.30 ±	0.05_c
tyr	3.45 ±	0.08_a	4.51 ±	0.01_b	3.75 ±	0.08_c
val	6.92 ±	0.03_a	10.18 ±	0.15_b	8.62 ±	0.16_c
met	1.94 ±	0.06_a	2.75 ±	0.01_b	2.13 ±	0.01_c
lys	3.53 ±	0.02_a	2.20 ±	0.09_b	3.19 ±	0.07_c
ile	4.54 ±	0.06_a	6.76 ±	0.12_b	6.08 ±	0.16_c
leu	8.39 ±	0.00_a	11.77 ±	0.05_b	10.13 ±	0.07_c
phe	5.31 ±	0.10_a	6.80 ±	0.35_{ab}	6.20 ±	0.12_b

* Asx includes aspartate + asparagine and Glx includes glutamine + glutamate. Different letters represent significant difference ($p < 0.05$).

Table 2
Identification of amino acid sequences from the most potent oat peptide fractions by LC-MS/MS analysis with average local confidence >90%.

Fraction	Peptide	Tag Length	ALC (%)	*m/z*	RT	Mass
1 kDa	LPVDVL	6	97	655.44	18.71	654.40
	LPKYQ	5	96	648.37	13.5	647.36
	LPPQL	5	96	567.38	15.73	566.34
	E(-18.01) LFGK	5	95	575.32	22.42	574.31
	APGAGVY	7	95	634.35	13.21	633.31
	LPQYQ	5	95	648.37	13.58	647.33
	FPLLQ	5	94	617.39	18.14	616.36
	FPTLN	5	94	591.35	16.57	590.31
	FPLLF	5	94	636.41	22.44	635.37
	FPLLN	5	94	603.38	17.95	602.34
	LLVVLL	6	92	669.46	19.89	668.48
	FPLLL	5	92	602.43	21.69	601.38
	LPAL	4	91	413.30	16.27	412.27
	LPVL	4	90	441.34	17.41	440.30
	LSPLF	5	90	576.38	18.58	575.33
F2 (1–5 kDa)	LPPQL	5	93	567.41	14.70	566.34
	FPLLQ	5	92	617.44	17.73	616.36
	LPELQ	5	91	599.39	14.44	598.33
F3 (1–5 kDa)	GDVVALPA	8	*	741.48	16.12	740.41
	DVVALPAG	8	*	741.48	14.85	740.41
	YPTNTY	6	95	758.41	13.09	757.33
	DFPVY	5	94	640.35	18.42	639.29
	E(-18.01) VFGK	5	92	561.31	19.11	560.30
	LPVDV	5	91	542.33	15.24	541.31
	LPLPQ	5	90	567.37	18.05	566.34

the C-terminus and/or Met or Ala at the ultimate chain's position would contribute to the α-glucosidase inhibitory effect (Ibrahim et al., 2018). Sequences such as Tyr-Pro-Thr-Asn-Thr-Tyr (YPTNTY) and Gly-Asp-Val-Val-Ala-Leu-Pro-Ala (GDVVALPA), found in oat protein, contained Tyr in both sides of the chain, and Thr, Ala, Pro in the suggested position and near the middle of the structure as pointed by Nishioka, Watanabe, Kawabata, and Ryoya (1997).

Diprotin A and B are strong DPP-IV inhibitors with IC_{50} values of 1.1 and 5.5 μg/mL with amino acid sequences: Ile-Pro-Ile and Val-Pro-Leu, respectively, as reported by Umezawa et al. (1984). Interestingly, de novo peptide sequencing of F3 disclosed the presence of sequences such as Leu-Pro-Val-Asp-Val (LPVDV), Leu-Pro-Leu-Pro-Gln (LPLPQ), and Tyr-Pro-Thr-Asn-Thr-Tyr (YPTNTY) (Table 2), which resemble the known inhibitors in that they both contain Val, Pro, and Leu in their N-terminal structures, and Pro in the chain's second position. In addition, dipeptides Leu-Pro and Ile-Pro have been demonstrated to be some of the main DPP-IV inhibitors present in rice bran, and Tyr-Pro was found as an inhibitor in milk protein (Hatanaka et al., 2012; Nongonierma & Fitzgerald, 2014). Therefore, we can speculate that the presence of these dipeptides in oat peptide sequences such as Gly-Asp-Val-Val-Ala-Leu-Pro-Ala (GDVVALPA) and Asp-Val-Val-Ala-Leu-Pro-Ala-Gly (DVVALPAG) might have significantly contributed to the AFH antidiabetic activity. Studies on binding modes of DPP-IV inhibitors to their active site showed that the presence of rings gave rigidity to gliptins structures; for instance, the cyclopropane component to the general cyanopyrrolidine structure allowed saxaglipting to hydrophobically interact at the S1 subsite of the enzyme (Nabeno et al., 2013). It is known that the ligand sitagliptin mechanism of action consists of hydrogen bonding and hydrophobic interactions at the active site of the enzyme, which gives it the characteristic of a competitive inhibitor (Chakraborty et al., 2014; Nongonierma & FitzGerald, 2019). Some of the peptide structures obtained (supplementary data) may have contributed to the inhibitory activity due to the presence of rings and bulky groups that could provide rigidity to the peptide structures for better interaction with DPP-IV. However, further studies are required to address the kind of interactions that the obtained peptides exert over DPP-IV.

Due to its abundance in oat protein, Pro content is high in the identified sequences in our study. It is possible that those peptides with antidiabetic effects could better resist gut enzymatic digestion; thus, maintaining their stability and bioavailability. Yet, the release of the smaller peptides from the identified sequences in this study after gut digestion needs to be investigated, as well as their antidiabetic activities. Moreover, the peptides with antidiabetic activities identified have a relatively small M_W, with most of them having a 5 amino acid length. Thus the absorption of those peptides is possible through specific peptide transporters, paracellular transport, or transcytosis route (Oseguera-Toledo, González de Mejía, Reynoso-Camacho, Cardador-Martínez, & Amaya-Llano, 2014). Other studies using molecular docking, cell culture and *in vivo* models are required to understand the underlying mechanisms of oat peptides digestion, absorption, and interactions with DPP-IV.

4. Conclusion

This study found that peptides from oat protein, through continuous hydrolysis using alcalase and flavourzyme, inhibited enzymes like DPP-IV, α-amylase, and α-glucosidase. Ultrafiltration and RP-HPLC fraction techniques were effective to concentrate DPP-IV and α-amylase inhibitory peptides from low M_W hydrolysates. LC-MS/MS analysis disclosed the presence of two eight amino acid sequences from the most effective fractions, identified as GDVVALPA and DVVALPAG, as well as 25 new de novo sequences rich in hydrophobic and aromatic amino acids and proline. The results suggest that proline and hydrophobic amino acids play a crucial inhibitory role and may favor hydrophobic interactions and hydrogen bonding at the active site of these enzymes. Although the information in this research might seem basic, it is the first necessary stage to develop peptides with antidiabetic activities from oat as a relatively new source of protein of plant origin. Our data indicate that it is possible to generate antidiabetic peptides from oat protein that targets three of the most studied enzymes for glucose regulation, which casts a new light for the study of oat peptides and their future in pharma and nutraceutical applications for T2DM management. This research also justifies studying the antidiabetic effects of peptides through cell and animal models in the future and understanding their interactions and mechanisms of action. It should be mentioned that optimization of the processing is required in terms of enzyme addition level and reaction time in the future to generate peptides for functional food applications once the peptide antidiabetic activities are validated in both cell and animal models. This knowledge may allow the industry to implement bioactive peptides in diabetic friendly foods and general products in addition to oat β-glucans and phenolic compounds and add value to oats as a globally beneficial crop.

5. Ethics statement

The aim of the present work was to investigate how different enzymes were inhibited by oat peptides obtained through alcalase and flavourzyme treatment. Therefore, the research did not include any human subjects and/or animal experiments.

CRediT authorship contribution statement

Lourdes Ramirez Fuentes: Investigation, Methodology, Formal analysis, Writing – review & editing. **Caroline Richard:** Writing – review & editing, Visualization. **Lingyun Chen:** Supervision, Conceptualization, Writing – review & editing, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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