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Identification and *in silico* study of a novel dipeptidyl peptidase IV inhibitory peptide derived from green seaweed *Ulva* spp. hydrolysates

Raúl E. Cian [a,*], Agustina E. Nardo [b], Antonela G. Garzón [a], María C. Añon [b], Silvina R. Drago [a]

[a] Instituto de Tecnología de Alimentos, CONICET, FIQ - UNL, 1° de Mayo 3250, (3000), Santa Fe, Argentina
[b] Laboratorio de Investigación, Desarrollo e Innovación en proteínas Alimentarias (LIDiPA), Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), Comisión de Investigaciones Científicas (CIC-PBA) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET- CCT La Plata), Universidad Nacional de La Plata (UNLP), CIDCA calle 47 y 116, 1900, La Plata, Argentina

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ABSTRACT

A number of functional foods containing seaweed-derived peptides are currently commercialized. Some seaweed derived peptide-containing products include Wakame peptide® and Nori peptide S®. To develop functional foods with the addition of *Ulva* spp. peptides, the isolation and identification of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides from hydrolysates of this seaweed was performed. *Ulva* spp. peptides were fractionated and purified sequentially by anion exchange, ultrafiltration, and reversed phase high performance liquid chromatography. The fractions obtained from each analytical step were collected and the *in vitro* enzyme inhibitory activity was evaluated. The applied purification process increased the *in vitro* DPP-IV inhibitory activity. The inhibition mechanism of DPP-IV of the most active fraction was evaluated and it was analyzed by MALDI-TOF. A peptide SLAVSVH was identified. It has 57% of hydrophobic residues in their sequence (including Ala and branched chain amino acids), which could be a common feature among inhibitory peptides of DPP-IV. Molecular docking analysis showed that SLAVSVH/DPP-IV complex was stabilized by CH–π interactions between the side chains of Val6 with the indol of Trp629, and between rings of His7 with Trp563. The energy values obtained for the peptide under study indicate that it is a good candidate to inhibit DPP-IV.

1. Introduction

Chlorophyta (green) and *Rhodophyta* (red) macroalgae have a high protein content and are a promising source for the development of functional foods (Garcia-Vaquero & Hayes, 2016; Øverland, Mydland, & Skrede, 2019). Among the edible green macroalgae the genus *Ulva* are widely distributed along the coasts of the world's oceans (Cian et al., 2018). *Ulva* spp. has a protein content ranging from 15 to 35%, making it a good protein source for producing bioactive peptides (Garcia-Vaquero & Hayes, 2016; Harnedy & FitzGerald, 2011). This allows the generation of new bioactive peptides from edible green seaweeds (Admassu, Gasmalla, Yang, & Zhao, 2018). In this regard, many *Ulva*-derived bioactive peptides have been obtained by *in vitro* enzymatic hydrolysis (Cian et al., 2018; Garcia-Vaquero, Mora, & Hayes, 2019; Pimentel, Alves, Harnedy, & FitzGerald, 2019). These peptides have shown to exert many bioactive properties such as: antioxidant, anticancer, antihypertensive, and immunomodulatory (Cian et al., 2018; Lafarga, Acién-Fernández, & Garcia-Vaquero, 2020; Fan, Bai, Zhu, Yang, & and Zhang, 2014). On the other hand, different seaweed-derived bioactive peptides have shown to inhibit the enzyme dipeptidyl peptidase IV (DPP-IV) (Shannon, Conlon, & Hayes, 2021). However, for *Ulva* spp., the isolation and identification of DPP-IV inhibitory peptides has not been studied.

DPP-IV (EC 3.4.14.5) is a serine protease that modulates the biological activity of specific circulating peptide hormones by specifically cleaving 2N-terminal amino acids: Xaa-Pro and Xaa-Ala (Hatanaka et al., 2012). DPP-IV acts to inactivate two incretin hormones that enhance insulin secretion, GLP-1 and glucose-dependent insulinotropic peptide. Therefore, inhibition of DPP-IV is a molecular target in diabetes treatment (Yan, Zhao, Yang, & Zhao, 2019). In this regard, DPP-IV inhibitors have been used as drugs to control postprandial glycemia in type 2 diabetes mellitus (Hatanaka et al., 2012). DPP-IV accepts a wide variety of inhibitors of different chemical structure (Nojima et al., 2016), probably because its active center is located in a long cavity (≥20 Å). The way of binding of different inhibitors has been extensively studied and different subpockets, S1, S2, S1′ and S2' have been described by means of which the substrates and competitive inhibitors establish their

* Corresponding author.
E-mail address: recian@fiq.unl.edu.ar (R.E. Cian).

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unions. While S1 (Tyr 631, Val656, Trp659, Tyr662, Val711) is primarily hydrophobic, S2 (Arg125, Glu205, Glu206, Phe357, Ser209, Arg358) is a primarily charged subpocket; the Glu205 and Glu206 residues have been reported to be key to targeting the N-terminus of DPP-IV-substrates for its correct interaction with the Ser630 by salt bridge interactions (Juillerat-Jeanneret, 2014). Although most synthetic DPP-IV inhibitors are generally well-tolerated, some side effects have been recently reported, including naso-pharyngitis, headaches, and urinary infections (Jao et al., 2015). Therefore, it is important for type 2 diabetes mellitus therapy to identify DPP-IV inhibitor peptides from natural sources and then develop bio-functional foods that allow reducing the use of synthetic inhibitors.

The aims of this work were: i) to evaluate the capacity of *Ulva* spp. protein hydrolysate to inhibit DPP-IV, ii) to isolate the peptides from *Ulva* spp. protein hydrolysate by chromatographic techniques and ultrafiltration process, and iii) to identify the peptides responsible for this activity using MALDI-TOF tandem mass spectrometry. Furthermore, peptide characterization through *in silico* analysis was performed.

2. Material and methods

2.1. Reagents

Alkaline protease-Protex 6L® (AP) and Neutral protease-Purazyme® (NP) enzymes were provided by DuPont (Buenos Aires, Argentina) and Nutring (Buenos Aires, Argentina), respectively. Flavourzyme enzyme (F) (P6110), dipeptidyl peptidase IV enzyme (DPP-IV) (D4943), *o*-phthaldialdehyde (P1378), Gly-Pro-*p*-nitroanilide (G0513), and alpha-cyano-4-hydroxy cinnamic acid (233099) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Molecular weight standards conalbumin, carbonic anhydrase, cytochrome C, aprotinin, bacitracin, cytidine, and glycine were obtained from Pharmacia Fine Chemicals (Piscataway, NJ, USA). AG-X4 resin (100–200 mesh) was obtained from Biorad® (California, USA). Trifluoroacetic acid (TFA) and acetonitrile HPLC grade were obtained from Merk® (Buenos Aires, Argentina). Tris buffer, sodium acetate buffer, sodium chloride, sodium hydroxide, hydrochloric acid, sulfuric acid, acetone, and boric acid analytical grade were obtained from Cicarelli Laboratorios (San Lorenzo, Santa Fe, Argentina).

2.2. Raw materials

One kilogram of different specimens of *Ulva* spp. was handpicked in Punta Maqueda (Comodoro Rivadavia, Argentina). The seaweed was processed according to Cian et al. (2018). Briefly, *Ulva* spp. was dispersed at 25 g kg^{-1} in acetone for 30 min and filtered through a 50-mesh sieve (0.297 mm). The residue was re-extracted four times. To remove the remained solvent, the residue was dried using ARCANO DFZ-6020 (China) vacuum oven at 40 °C for 3 h. The dry residue obtained without chlorophyll was used as substrate for enzymatic hydrolysis.

2.3. Ulva spp. hydrolysates

Ulva spp. hydrolysates were obtained according to Cian et al. (2018). The enzymatic systems used to obtain the hydrolysates were the following:

- Hydrolysate NPF: hydrolysis with NP enzyme 2 h + hydrolysis with F enzyme during 2 h; total reaction time, 4 h.
- Hydrolysate APF: hydrolysis with AP enzyme 2 h + hydrolysis with F enzyme during 2 h; total reaction time, 4 h.

For hydrolysis with NP + F system, the working conditions were: temperature 50 °C, pH 7.0, enzyme/substrate (E/S) ratio 5 g 100 g^{-1}, for both NP and F enzymes. For hydrolysis with AP + F system, the working conditions were: temperature 55 °C, pH 9.5, enzyme/substrate (E/S) ratio 5 g 100 g^{-1} and temperature 50 °C, pH 7.0, enzyme/substrate (E/S) ratio 5 g 100 g^{-1}, for A and F respectively.

Free amino groups were measured using *o*-phthaldialdehyde, according to Nielsen, Petersen, and Dambmann (2001), and the degree of hydrolysis (DH) was calculated as:

$$DH = [(h - h_0) / h_{tot}] \times 100$$

Where h_{tot} is the total number of peptide bonds in the protein substrate (8.04 mEq g^{-1} proteins); h is the number of peptide bonds cleaved during hydrolysis, and h_0 is the content of free amino groups of substrate.

Protein and solid contents of hydrolysates were determined according to the AOAC (2000) methods.

Fractionation by gel filtration chromatography of hydrolysates was performed according to Cian, Garzón, Betancur-Ancona, Chel-Guerrero, and Drago (2015). 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 evaluation of DPP-IV inhibitory activity of *Ulva* spp. hydrolysates was performed according to Wang et al. (2017). 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. Briefly, 25 μL of samples at 6 mg mL^{-1} protein in 100 mmol L^{-1} Tris buffer (pH 8.0) were added with 25 μL of 1.59 mmol L^{-1} Gly-Pro-*p*-nitroanilide in Tris buffer and incubated at 37 °C for 20 min. Then, 50 μL of 0.01 U mL^{-1}DPP-IV were added and the reaction mixture was incubated at 37 °C for 60 min. The reaction was stopped by adding 100 μL of 1 mol L^{-1} sodium acetate buffer (pH 4.0). The absorbance of the resulting solution was measured at 385 nm with an Asys UVM 340 Microplate Reader (BMG LABTECH, Offenburg, Germany). The DPP-IV inhibition rate was calculated as follows (1):

$$\text{DPP-IV inhibition: } [(A_E - A_{RB}) - ((A_S - A_{BS}) - A_{RB}))] \times 100 / (A_E - A_{RB}) \quad (1)$$

Where: A_E: is the absorbance of the enzyme, A_{RB}: is the absorbance of the reagent blank, A_S: is the absorbance of the sample, and A_{BS}: is the absorbance of the blank sample.

The concentration of sample needed to inhibit 50% DPP-IV activity was defined as IC_{50} value. To determine the IC_{50}, serial dilutions of samples from 0 to 6 mg mL^{-1} of protein were prepared. The experimental data were fitted with Origin software version 7.5 (OriginLab®, Northampton, Massachusetts, USA) using the following equation (2):

$$y = y_0 + A^{*}\exp(R_0{*}x) \quad (2)$$

Where: y, is the inhibition rate, y_0, A, and R_0 are the regression parameters, and x is the peptide concentration (mg mL^{-1} protein). The IC50 value was obtained as (3):

$$IC_{50} = \ln[(50\text{-}y_0/A)]/R_0 \quad (3)$$

All determinations were performed by triplicate.

2.4. Anionic exchange chromatography

The *Ulva* spp. hydrolysate was fractionated by anion exchange chromatography at pH 7.0 according to Gomez, Figueroa and Zapata (2013). For this, an AG-X4 resin (100–200 mesh) - Biorad® (USA) was used. Elution was performed with a linear gradient of NaCl (0.2, 0.4, and 0.6 mol L^{-1}) and was monitored by absorbance measurement at 280 nm. Four fractions (F_1, F_2, F_3 and, F_4) were obtained according to the concentrations of NaCl used in the gradient. The first fraction was eluted with distilled water (F_1), while the others were obtained with 0.2, 0.4 or

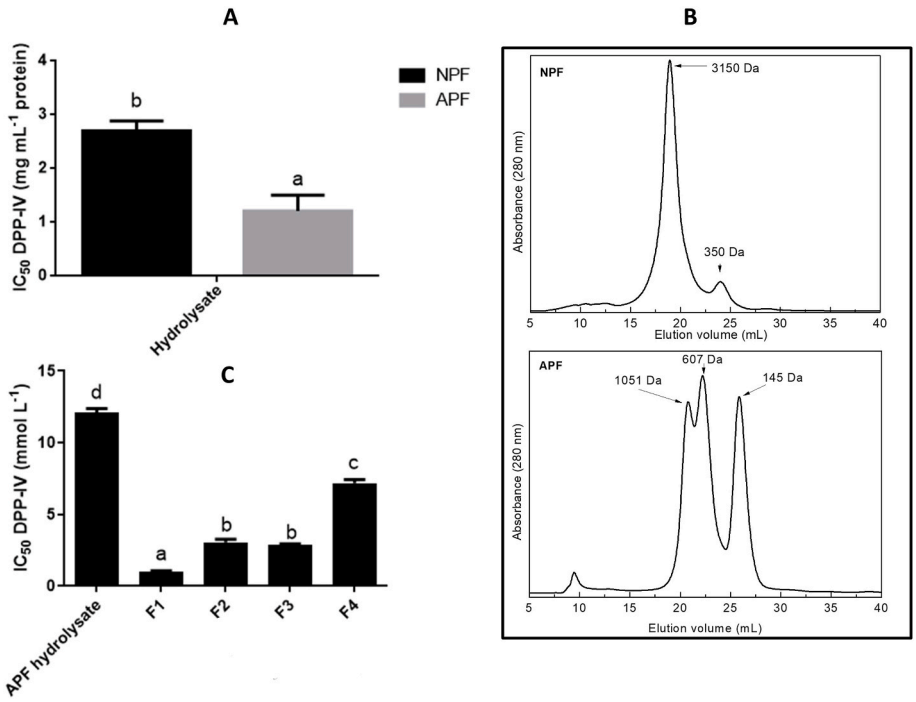

Fig. 1. Inhibition of dipeptidyl peptidase IV enzyme (DPP-IV) by NPF and APF hydrolysates obtained from *Ulva* spp. (**A**); FPLC profile of NPF and APF hydrolysates (the molecular sizes of the most representative peaks of the collected fractions are shown) (**B**); inhibition of DPP-IV enzyme by the anionic exchange chromatography fractions (**F1–F4**) obtained from APF hydrolysate (**C**). Bars with different letters indicate significant differences ($p < 0.05$).

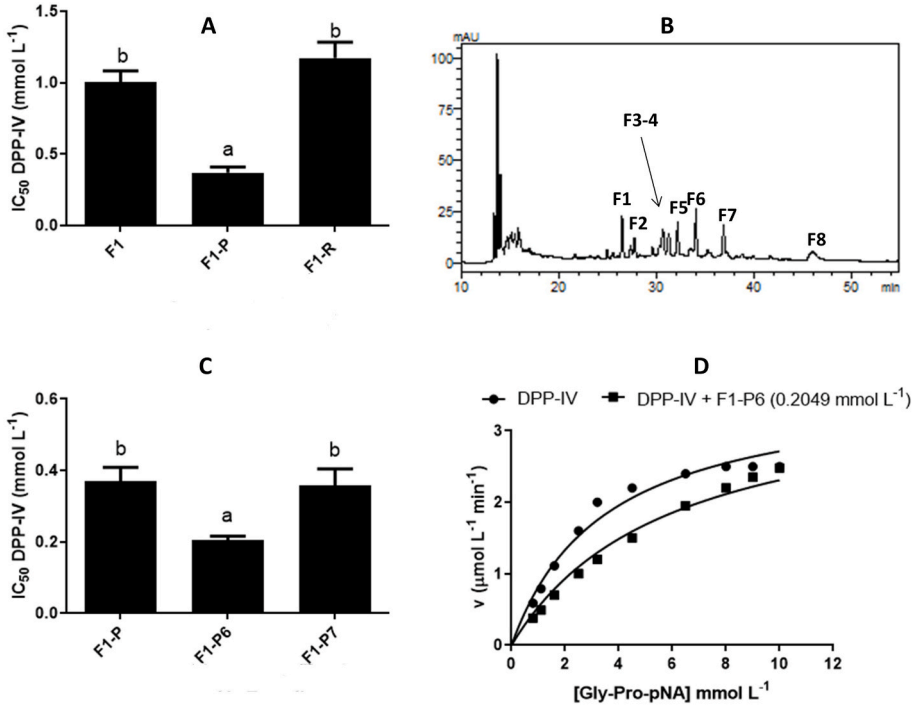

Fig. 2. Inhibition of dipeptidyl peptidase IV enzyme (DPP-IV) by ultrafiltered fractions (**F1–P: permeate** and **F1-R: retentate**) obtained from F1 fraction (**A**); RP-HPLC profile of the fraction **F1–P** at 220 nm and 280 nm (**B**); inhibition of DPP-IV enzyme by RP-HPLC fractions (**F1–P1 – F1–P8**) obtained from **F1–P** fraction (**C**); Michaelis-Menten plots of DPP-IV in absence (●) or presence of F1–P6 fraction (■) (**D**). Bars with different letters indicate significant differences ($p < 0.05$).

0.6 mol L^{-1} NaCl, respectively. The peptide content of each fraction was determined by the method proposed by Nielsen et al. (2001), and the value was expressed as mmol L^{-1}. The DPP-IV inhibition assay was performed as described before. To determine the IC_{50}, serial dilutions of sample from 0 to 15 mmol L^{-1} of peptide content were made. The experimental data were fitted with Origin software version 7.5 (OriginLab®, Northampton, Massachusetts, USA) using equation (2). All determinations were performed by triplicate.

Table 1
Enzyme kinetic analysis of DPP-IV enzyme inhibited by F1–P6 fraction.

	Peptide concentration (mmol L^{-1})	K_m^{app} (mmol L^{-1})	V_{max}^{app} (μmol min^{-1} L^{-1})
DPP-IV	0	3.498 ± 0.097[a]	3.664 ± 0.094[a]
DPP-IV + F1–P6 fraction	0.204	7.415 ± 0.101[b]	3.582 ± 0.105[a]

K_m^{app}: Michaelis constant. **V_{max}^{app}**: maximum reaction velocity. Results are expressed as mean value ± standard deviation. Different letters in the same column mean significant differences among samples ($p < 0.05$) according to least significant difference (LSD) test.

2.5. Ultrafiltration process

The most bioactive fraction from APF hydrolysate obtained by anion exchange chromatography was selected to continue the purification process. The fraction F_1 was ultrafiltered using a 1 kDa cut-off Molecular/Por Cellulose-Ester membrane and Molecular/Por Stirred Cell S-43-70 system (Interchim, Montluçon, France). The volume reduction factor was 4. The two fractions obtained by ultrafiltration process from F_1 were named: F_1-R (>1 kDa) and F_1–P (<1 kDa). The peptide content of each fraction was determined by the method proposed by Nielsen et al. (2001), and the value was expressed as mmol L^{-1}. The DPP-IV inhibition assay and the IC_{50} value were determined as described in 2.4 but using serial dilutions of sample from 0 to 1.5 mmol L^{-1} of peptide. All determinations were performed by triplicate.

2.6. Reversed phased - high performance liquid chromatography (RP-HPLC)

The most bioactive fraction obtained from ultrafiltration process (F_1–P) was selected to continue the purification process. RP-HPLC fractionation process was performed according to Zhang et al. (2013) with modifications. The HPLC system consisted in a Shimadzu Series LC-20AT pump, with Shimadzu SPDM20A diode array detector, equipped with a Phenomenex C18 column (Gemini 250 × 4.6 mm, 5 μm) (Thermo Fisher Scientific, USA). Elution was performed by gradient, using 0.1 g trifluoroacetic acid (TFA) in 100 mL water as mobile phase A, and acetonitrile with 0.1 g 100 mL^{-1} TFA as mobile phase B. The gradient increased linearly, from 0 g 100 mL^{-1} to 40 g 100 mL^{-1} of B, in a run time of 60 min. The column was placed at 40 °C, and the elution flow was 1 mL min^{-1}. Fractions that gave absorbance peaks at 220 nm and/or 280 nm were collected. The fractions obtained by RP-HPLC process from F_1–P were named: F_1–P_1, F_1–P_2, F_1–P_3, F_1–P_4, F_1–P_5, F_1–P_6, F_1–P_7, and F_1–P_8. The peptide content of each fraction was determined by the method proposed by Nielsen et al. (2001), and the value was expressed as mmol L^{-1}. The DPP-IV inhibition assay and the IC_{50} value were determined as described in 2.4 but using serial dilutions of sample from 0 to 0.6 mmol L^{-1} of peptide. All determinations were performed by triplicate.

2.7. Enzyme kinetic analysis

The kinetic analysis of DPP-IV enzyme and the DPP-IV added with the most bioactive RP-HPLC fraction (F_1–P_6) was performed using Michaelis-Menten equation. For this, different substrate concentrations (0.8–10 mmol L^{-1} Gly-Pro-*p*-nitroanilide) were incubated with DPP-IV solution with and without peptide fraction at their IC_{50} concentration (0.2049 mmol L^{-1}). The DPP-IV inhibition assay of each system was performed as described before according to Wang et al. (2017). Then,

Table 2
Molecular docking results.

Peptide	Autodock	FlexPepDock[b]	
	Binding energy[a] (Kcal mol^{-1})	Rosetta Score	Interface energy (Kcal mol^{-1})
IPI (control)	−10.32	−1402 ± 6	−9 ± 1
SLAVSVH	−7.65	−1392 ± 4	−15 ± 2

[a] Binding energy estimated with Lamarckian genetic algorithm of AutoDock.
[b] Rosetta score and interface energy obtained by FlexPepDock with Autodock models.

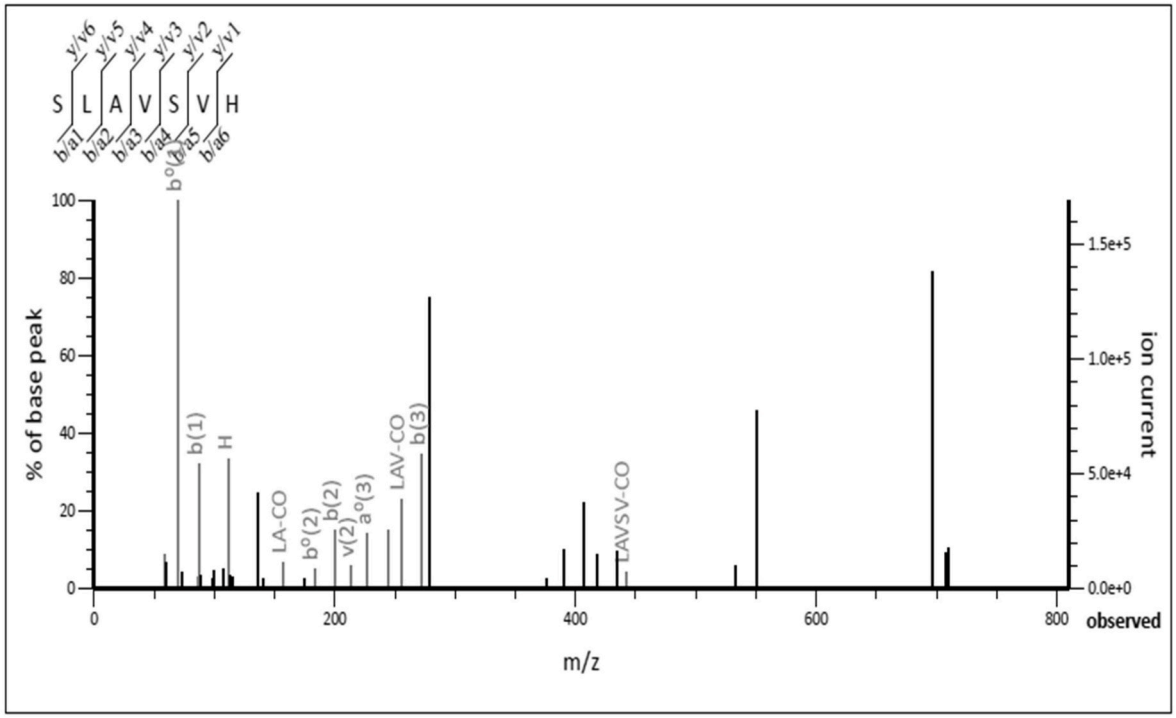

Fig. 3. Fragmentation spectrum of the peptide identified from the most bioactive fractions (F1–P6).

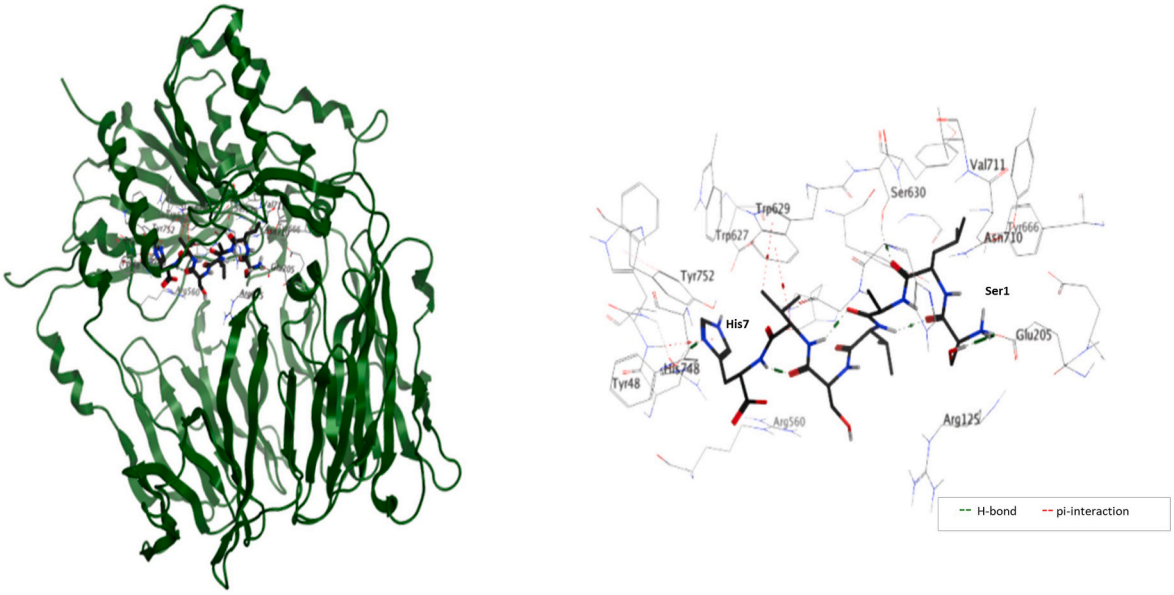

Fig. 4. Conformation details at the active site of DPP-IV with SLAVSVH of the best model of by FlexPepDock.

the experimental data were fitted with GRAPHPAD PRISM software version 6.07 (GraphPad, Software Inc., San Diego, CA, USA) using Michaelis-Menten equation. V_{max} and K_m parameters were provided by the same software taking into account all the experimental plots. All determinations were performed by triplicate.

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

The most bioactive RP-HPLC fraction (F_1–P_6) was also selected to identify the peptide responsible for the activity using MALDI-TOF tandem mass spectrometry. For this, 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, Kyoto, Japan) equipment. The determinations were made in reflectron mode. Once selected for MS/MS fragmentation, precursor ions were subjected to true high energy collisions with the collision gas (helium). The resulting fragment ions were analyzed in the second TOF region incorporating the curved field reflectron and detected with the ultrafast MCP detector (Shimadzu, Kyoto, Japan). 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 ($p < 0.05$).

2.9. In silico analysis

Studies *in silico* of the peptide obtained were conducted. The pI and hydrophobicity were determined using the PepDraw program (http://www.tulane.edu/~biochem/WW/PepDraw/), and a simulation of the gastrointestinal digestion of the peptide was performed using the BIOPEP-UVW database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep).

2.10. Molecular docking

Structure of SLAVSVH was generated with PepFold3 (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/). The crystal structure of human DPP-IV complex with a peptide inhibitor (IPI, named Diprotin A) (PDB ID 1WCY) was obtained from Protein Data Bank (http://www.rcsb.org). Docking studies were performed in two steps. First analysis was performed using AutoDock Tools 4.2.6 and AutoDock 4.0 (Script Rearch Institute, La Jolla, CA, USA). Before the docking one of the enzyme subunit was selected and water molecules were removed. The polar hydrogens were then added according to the specifications of the AutoDock developers. The binding site for docking was created around at the IPI binding site on the crystal (x: 57.80, y: 63.00, z: 37.50) with a cube geometry with an edge of 30 Å in order to include the entire cavity binding. Lamarckian genetic algorithm (LGA) was performed for conformational search. The number of docking runs was set to 50 with a population size of 300 and a maximum of 2500000 energy evaluations. The best ranked docking pose of peptides in the active site of DPP-IV was obtained according to the scores and binding energy value (Morris et al., 2009). Diprotin A, a peptidic inhibitor of DPP-IV was docked as a positive control. The complexes obtained were superimposed with crystal structure obtaining a root-mean-square deviation of atomic positions (RMSD) value of 0.7 Å.

In a second stage, interaction energy calculations were refined starting from the best docking conformation obtained with Autodock using FlexPepDock server (http://flexpepdock.furmanlab.cs.huji.ac.il/) (London, Raveh, Cohen, Fathi, & Schueler-Furman, 2011). This server is based on Rosetta modeling software (version 3.2) and it was employed to find high-resolution modeling of protein-peptide interactions allowing full flexibility to the peptide and side-chain of the receptor (London et al., 2011). Starting from the same complex, three independent refinements were made. The best models of each refinement were select to analyzed interactions and binding modes between ligands and DPP-IV. Visual analysis was done using VMD (Humphrey, Dalke, & Schulten, 1996).

2.11. Statistical analysis

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 α: 0.05, using the STATGRAPHICS Centurion XV 15.2.06 (StatPoint Technologies, Inc., Warrenton, Virginia, USA).

3. Results and discussion

3.1. Characterization and DPP-IV inhibitory activity of hydrolysates

Ulva spp. hydrolysates were obtained with two different enzyme sequential systems Neutral protease + Flavourzyme (NPF) and Alkaline protease + Flavourzyme (APF). The degree of hydrolysis (DH) of APF was higher than that obtained for NPF (21.8 ± 1.8 mEq 100 total mEq^{-1} *vs*. 15.0 ± 1.0 mEq 100 total mEq^{-1}, respectively) ($p < 0.05$), indicating that the APF was more efficient on *Ulva* spp. protein cleavage. Similar results were reported by Cian et al. (2018) for *Ulva* spp. hydrolysates obtained with the same sequential hydrolysis systems (DH of 23.6 ± 2.2 mEq 100 total mEq^{-1} and 16.7 ± 1.7 mEq 100 total mEq^{-1} for alkaline protease + Flavourzyme and neutral protease + Flavourzyme, respectively).

As shown in Fig. 1A, both *Ulva* spp. hydrolysates inhibited the DPP-IV enzyme. The IC_{50} values obtained by NPF and APF were 2.70 ± 0.19 mg mL^{-1} and 1.21 ± 0.12 mg mL^{-1}, respectively. These results are similar to those reported for defatted rice bran (2.3 ± 0.1 mg mL^{-1}, Hatanaka et al., 2012), *Palmaria palmata* (1.65 ± 0.12 mg mL^{-1}, Harnedy & FitzGerald, 2013), and whey (1.00–1.43 mg mL^{-1}, Nongonierma & FitzGerald, 2013) protein hydrolysates. Moreover, APF had lower IC_{50} value than NPF (Fig. 1A), meaning greater inhibitory activity against DPP-IV enzyme. Note that IC_{50} value of APF was lower than that reported by Harnedy, O'Keeffe, and FitzGerald (2015) for red seaweed *Palmaria palmata* protein hydrolysate (1.47 ± 0.09 mg mL^{-1}), indicating that APF is a good source of DPP-IV inhibitory peptides.

Wang et al. (2017) reported that the DPP-IV inhibition rate increased with higher DH, indicating that the most active peptides are those of low molecular weight (MW). In line with this, APF showed higher proportion of low MW components than NPF. Fig. 1B shows that APF presented three main peaks: 1051 Da, 607 Da, and 145 Da. The peak of 607 Da could correspond to low MW peptides, representing ≈43% of the total area of the chromatogram. However, the peak of low MW peptides in NPF (350 Da) represented ≈13% of this total area. These results are in agreement with those reported by Hatanaka et al. (2012), who found that defatted rice bran hydrolysate obtained with Umamizyme G exhibited lower IC_{50} value for DPP-IV enzyme than that produced with Bioprase SP. Moreover, the FPLC profile of Umamizyme G hydrolysate showed a high proportion of low MW peptides with a main peak of ≈300 Da. On the other hand, the peak of 1051 Da in APF profile corresponds to intermediate MW species, while 145 Da peak correspond to free amino acids (Fig. 1B).

Taking into account these results, APF hydrolysate was selected for further peptide purification process.

3.2. Peptide purification

All fractions obtained through anion exchange chromatography from APF hydrolysate were able to inhibit the DPP-IV activity (Fig. 1C). Among them, F_1 showed the lowest IC_{50} value ($p < 0.05$). This result indicates that peptides from APF hydrolysate eluted with distilled water have the highest inhibitory effect on DPP-IV enzyme. These peptides were not retained by the column, since it exchanges anions. Thus, the presence of positively charged peptides at pH 7.0 in APF plays an important role in DPP-IV enzyme inhibition. Similar results were found by Lacroix and Li-Chan (2014) for bovine whey DPP-IV inhibitory peptides fractionated by cation exchange chromatography. These authors obtained seven peptide fractions that inhibited the DPP-IV enzyme. Among them, the fractions strongly retained by the column (positively charged peptides) were the most active. Different DPP-IV inhibitory peptides having basic amino acid residues in its sequence such as: His, Lys, Asn or Arg have been reported from several food protein sources (Nongonierma & FitzGerald, 2017). Mudgil, Kamal, Yuen, and Maqsood (2018) isolated a novel anti-diabetic and anti-obesity peptide (FCLPLPLLK) from camel milk protein hydrolysate. Moreover, Liu et al. (2017) identified a DPP-IV inhibitory peptide from a marine bivalve mollusc (*Ruditapes philippinarum)* hydrolysate with a histidine at the C-terminus (FLMESH). Other DPP-IV inhibitory peptides with basic amino acid residues from milk proteins have also been reported such as: HL, WK, WN, and VR (Nongonierma & FitzGerald, 2013; Yan et al., 2019).

Based on the results obtained, the F_1 fraction from APF hydrolysate was fractionated by ultrafiltration. As shown in Fig. 2A, the most active fraction (lower IC_{50} value) was found in permeate (F_1–P) indicating that peptides with MW lower than 1000 Da were responsible for the inhibitory activity on DPP-IV. These results agree with those obtained by Oseguera-Toledo, Gonzalez de Mejia, and Amaya-Llano (2015) for DPP-IV *Phaseolus vulgaris* inhibitory peptides. These authors reported that the highest DPP-IV inhibitory activity was found in fractions with MW lower than 1000 Da. Moreover, the highest DPP-IV inhibitory activity was associated to lower 2000 Da MW peptides from milk proteins hydrolysates (Nongonierma & FitzGerald, 2013). In this regard, several authors have reported that peptides <1000 Da obtained from different protein sources (rice bran, amaranth proteins, ham, soybean, and fish proteins) have very good DPP-IV inhibitory activity (González-Montoya, Hernández-Ledesma, Mora-Escobedo, & Martínez-Villaluenga, 2018; Jao et al., 2015; Lu et al., 2019).

The most bioactive fraction obtained from ultrafiltration process (F_1–P) was fractioned by RP-HPLC obtaining eight novel peptides fractions (Fig. 2B). Surprisingly, only the fractions F_1–P_6 and F_1–P_7 had DPP-IV inhibitory activity (Fig. 2C). Moreover, F_1–P_6 showed the lowest IC_{50} value (0.2049 ± 0.0012 mmol L^{-1}). It is noteworthy that this fraction had considerable retention time in the RP-HPLC process indicating a relatively high hydrophobicity. Nongonierma and FitzGerald (2017) reported that hydrophobic amino acids such as Ala, Gly, Ile, Leu, Phe, Pro, Met, Trp, and Val are found within the DPP-IV inhibitory peptides and play an important role in the interaction with the active site of this enzyme. Moreover, *in silico* analyses have demonstrated that a hydrophobic N-terminal amino acid was related to DPP-IV inhibitory activity (Liu, Cheng, & Wu, 2019).

The enzyme kinetic analysis of DPP-IV and F_1–P_6 + DPP-IV were performed using Michaelis-Menten equation (Fig. 2D). As shown in Table 1, there was no significant difference in V_{max}^{app} without inhibitor or in the presence of F_1–P_6 ($p > 0.05$). However, the K_m^{app} value for DPP-IV activity was lower in the presence of peptide ($p < 0.05$), indicating that the DPP-IV reaction requires more substrate for catalysis in the presence of 0.204 mmol L^{-1} F_1–P_6. Moreover, an unaffected V_{max}^{app} indicates that the inhibitor competes with the substrate for the active site on the enzyme, which suggests that F_1–P_6 is a competitive inhibitor. Similar inhibition mode on DPP-IV enzyme was found for other peptide fractions obtained from different protein sources such as: whey protein (Nongonierma & FitzGerald, 2013), rapeseed (Xu et al., 2019), fish (Lacroix & Li-Chan, 2016), and casein (Nongonierma & FitzGerald, 2013). Zhang et al. (2016) isolated the competitive peptides HPINHR and GPFPILV from caprine casein, which presented DPP-IV-IC_{50} values of 452.2 ± 7.15 and 163.7 ± 1.33 μmol L^{-1}, respectively. Moreover, Nongonierma and FitzGerald (2013) reported that the di-peptides AL, FL and VA isolated from whey proteins were competitive inhibitors of DPP-IV, their DPP-IV-IC_{50} values being 882.1, 399.6 and 168.2 μmol L^{-1}, respectively. In this regard, the DPP-IV-IC_{50} value of F_1–P_6 was 204.9 ± 1.2 μmol L^{-1} which would indicate a good inhibitory activity among the peptides that act as competitive inhibitors of DPP-IV.

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 fraction F_1–P_6, analysis by MALDI-TOF tandem mass spectrometry was performed.

A search for MS/MS fragments in MASCOT allowed identifying one peptide (Fig. 3). The peptide sequence was **SLAVSVH** (mass/load (m/z): 711.81, +1). Moreover, the peptide identified was statistically significant according to MASCOT score histogram ($p < 0.05$), thereby confirming their identity in a 95% confidence.

The identified peptide has a residue of His. As mentioned above, different DPP-IV-inhibiting peptides have been identified with basic amino acid residues in their sequences (Nongonierma & FitzGerald, 2017). Moreover, the *in silico* isoelectric point of this peptide was 7.54, indicating that it had a positive net charge at pH 7.0 (working pH for the ionic exchange chromatography process). This result agrees with that obtained in anion exchange chromatography where the most active fraction was F_1. Note that the peptides in this fraction were not retained by the column.

Regarding the molecular size, the identified peptide had a MW within the range expected. Generally, the most active fraction against DPP-IV obtained from ultrafiltration process had a MW lower than 1000 Da. As mentioned before, the peptides reported to date with the ability to inhibit DPP-IV enzyme normally show low molecular size (Lu et al., 2019).

The identified peptide had 57% of hydrophobic residues in their sequence. Moreover, it was observed a high proportion of branched chain amino acids (≈43% of total amino acids) and a residue of Ala. This result matches with the relatively high retention time obtained in the separation by RP-HPLC process (Fig. 2B), indicating high hydrophobicity of this peptide. Moreover, *in silico* hydrophobicity of the identified peptide was +9.48 kcal mol^{-1}. Similar hydrophobicity results were reported by González-Montoya et al. (2018) for different DPP-IV inhibitory peptides. As mentioned before, it has been proposed that the presence of hydrophobic residues could be a necessary characteristic for DPP-IV inhibitory peptides (Nongonierma & FitzGerald, 2017). Also, some studies have reported that peptides containing Pro or Ala residues in their sequences could act as potent DPP-IV inhibitors (Cermeño et al., 2019).

On the other hand, some hydrophilic amino acids also seem to play an important role in the DPP-IV inhibition, since residues of Thr, His, Gln, Ser, Lys, and Arg have been found in sequence of DPP-IV inhibitory peptides (Nongonierma & FitzGerald, 2017). In this sense, the identified peptide had a Ser residue at the N-terminal position. Taga, Hayashida, Kusubata, Ogawa-Goto, and Hattori (2017) isolated a DPP-IV inhibitory tri-peptide with Ser residue at the N-terminal position (SPQ). Moreover, Nongonierma, Lamoureux, and FitzGerald (2018) reported a potent DPP-IV inhibitory peptide that had a serine residue at the same position (SPVVPF).

3.4. *In silico gastrointestinal digestion of peptide*

The *in silico* simulation of gastrointestinal digestion of the identified peptide using the hydrolysis tool of the BIOPEP-UWM program gave two new peptide products: **SL** and **AVSVH**. The di-peptide **SL** is listed in the BIOPEP-UWM database as DPP-IV enzyme inhibitor (peptide ID: 8560). This result could indicate that **SLAVSVH** could be hydrolyzed by digestive proteases (pepsin, trypsin, and chymotrypsin), generating a new peptide with DPP-IV inhibitory activity. Moreover, several DPP-IV inhibitory di-peptides are encrypted in **SLAVSVH** such as: AV, VS, SV, and VH (BIOPEP-UWM database, 2021). As previously mentioned DDP-IV releases dipeptides from the N-terminal of polypeptides and therefore the possibility that the sequence could be a substrate was analyzed. According to the reported substrates, human DPP-IV is capable of releasing the dipeptide Xaa-Pro and to a lesser extent Xaa-Ala (https://www.ebi.ac.uk/merops/cgi-bin/pepsum?id=S09.003, accessed June 2020); therefore SLAVSVH could not be cleaved by the enzyme.

3.5. *Molecular docking*

The probable mode of interaction of the SLAVSVH peptide with DPP-IV was further studied. The interaction energies are shown in Table 2. The complexes obtained were used as starting structures to refine the interaction energy calculations by using the FlexPepDock server. For the peptide control, the binding energy values do not differ significantly from that of SLAVSVH after refinement (Table 2). However, for SLAVSVH the refinement process increased the interaction energy. These results can be explained if the type of docking (rigid vs. flexible) carried out by the programs used is taken into account. Unlike the Autodock protocol, FlexPepDock performed considers the flexibility of the enzyme residues around the ligand and therefore gives a better approximation to the possible interactions, and consequently an energy calculation that will take into account interactions than in a rigid docking would be lost. This effect will also be influenced by the peptide length. The energy values obtained for the peptide under study indicate that it is a good candidate to inhibit DPP-IV.

As shown in Fig. 4, the peptide pose was stabilized by a salt bridge, hydrogen bonds and CH–π interactions. SLAVSVH orients its N-terminus to S2 subpocket, where Ser1 residue is capable of establish a salt bridge with the Glu205 and Glu206 of the enzyme, which would guide the carbonyl group of Leu2, to form a hydrogen bridge with Ser630. Finally, the complex is stabilized by CH–π interactions between the side chains of Val6 with the indol of Trp629, and between rings of His7 with Trp563.

4. Conclusions

A number of functional foods containing seaweed-derived peptides are currently commercialized. In order to develop these foods, it is necessary to be able to identify and characterize the most bioactive peptide fraction from seaweed hydrolysates. In this work, a peptide fraction containing an inhibitor of the dipeptidyl peptidase IV was identified from *Ulva* spp. hydrolysate after a fractionation and purification process. The identified peptide was SLAVSVH. The presence of hydrophobic and branched amino acids was consistent with their inhibitory activity against dipeptidyl peptidase IV enzyme. Moreover, the DPP-IV-IC_{50} value of peptide fraction was similar to that reported for other peptides isolated from food proteins that act as competitive inhibitors. The study of interaction between SLAVSVH and DPP-IV indicated that complex was stabilized by CH–π interactions between the side chains of Val6 with the indol of Trp629, and between rings of His7 with Trp563. Thus, SLAVSVH was a good inhibitor among the natural peptides and could be used as a bio-functional ingredient containing *Ulva*-derived peptides. Further studies should be carried out including the scaling up of the production of the fraction containing the SLAVSVH peptide and its subsequent evaluation in experimental animals.

Conflicts of interest

There are no conflicts to declare.

CRediT authorship contribution statement

Raúl E. Cian: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, and, Writing – review & editing. **Agustina E. Nardo:** Investigation, Methodology, Software, Validation, Writing – original draft. **Antonela G. Garzón:** Investigation. **María C. Añon:** Formal analysis, and, Writing – review & editing. **Silvina R. Drago:** Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Investigation, Methodology, Writing – original draft, and, Writing – review & editing.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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REC, AEN, and AGG carried out the experiment. REC, AEN, and SRD analyzed the data and wrote the paper, and had the primary responsibility for the final content. All the authors read and approved the final manuscript. The authors are thankful to PICT-2016-2879 from ANPCyT for the financial support.

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