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ARTICLE

Antihyperglycemic effect of a chicken feet hydrolysate via incretin system: DPP-IV-inhibitory activity and GLP-1 release stimulation

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The potential as natural dipeptidyl-peptidase IV (DPP-IV) inhibitor of hydrolysates of chicken feet proteins was investigated. Three hydrolysates were selected due to their high DPP-IV inhibitory capacity (>80% inhibition), showing IC_{50} values around 300 µg estimated protein/mL. One of them (named p4H) was selected for posterior analysis. Its effect on glucose tolerance test was assayed on two rat models of glucose-intolerance (diet and age-induced) and on healthy animals. 300 mg estimated peptide/kg body weight improved the plasma glucose profile in both glucose-intolerant models. It also stimulated active GLP-1 release in enteroendocrine STC-1 cells and in rat ileum tissue. In conclusion, our results point out that proteins of chicken feet are a good source of bioactive peptides as DPP-IV inhibitors. Moreover, our results highlight the potential of the selected hydrolysate (p4H) for the management of type 2 diabetes due to the dual function of inhibiting DPP-IV activity and inducing GLP-1 release.

Introduction

The prevalence of lifestyle-related diseases such as obesity and type 2 diabetes mellitus (T2DM) has become a healthcare problem in developed societies. Therefore, effective strategies are required to prevent the development of T2DM and the associated pathologies. A recent focus has pointed out to the biology of incretin hormones such as GIP and GLP-1 for glycemic regulation**.**

GLP-1 is an incretin hormone released from intestinal L-cells in response to nutrient ingestion, and that exerts glucoregulatory action through the stimulation of insulin secretion and the inhibition of glucagon secretion [1,2]**.** In addition to its insulinotropic actions, GLP-1 also improves beta-cell mass, delays gastric emptying, enhances satiety and reduces food intake [3,4]**.** Due to its physiological effects, a number of antidiabetic agents targeted towards the incretin system have emerged. These incretin therapies are mainly based on the use of GLP-1 mimetics, and also of dipeptidyl peptidase IV (DPP-IV) inhibitors, which protect from cleavage of active GLP-1 by DPP-IV. DPP-IV is a serine protease widely distributed among many tissues in the body and expressed as both membrane and soluble form in a variety of cell types. This enzyme principally cleaves proline or alanine containing dipeptides from the N-terminus of a polypeptide; thereby GLP-1 and GIP are potential DPP-IV targets. The concentration of GLP-1 in plasma rises rapidly after food intake but decreases due to its immediate degradation by DPP-IV. In recent years, DPP-IV inhibitors have been reported to prevent the cleavage of incretins by DPP-IV and to increase the half-life of the active hormones, and hence are available for management of glucose homeostasis [1]**.** Recently, another potential approach to improve glycemia has been postulated, which is the stimulation of the endogenous secretion of GLP-1 stored in L-cells [5].

The development of food protein-derived peptides has become a novel strategy for the prevention and management of T2DM. The hydrolysates rich in bioactive peptides are well metabolized, and confer fewer side effects than synthetic pharmaceutical drugs. Furthermore, there is a wide range of available underutilized resources of the food industry, which could be potential sources of bioactive peptides. Altogether, this could lead to expand the use of bioactive peptides in the nutraceutical food section [6]. Recent *in silico* analysis revealed that sequences are contained within dietary proteins that present structural features which make them natural precursors for generation of potent inhibitors of DPP-IV [7,8]**.** Previous studies have described that hydrolysates obtained from milk proteins [9,10], as well as other animal and vegetal sources [11–13] exhibit DPP-IV inhibitory activity *in vitro***.** However, only a few studies have evaluated the effects of biopeptides as DPP-IV inhibitors *in vivo*. Acute studies show that biopeptides can decrease plasma glucose concentration relative to the control in an oral glucose tolerance test (OGTT) in rodents [14,15]**.** And Mochida et al. showed that ZeinH (a hydrolysate prepared from corn zein) administration into the ileal loop produces an inhibition of DPP-IV activity in the ileal vein in rodents [16]**.**

Regarding stimulation of GLP-1 secretion by dietary proteins, it has been reported that meat hydrolysates stimulate GLP-1 release in the enteroendocrine cell lines NCI-H716 [17], STC-1 [18], and GLUTag [19] and in rodents [20]**.** Moreover, GLP-1 secretion triggered in response to whey protein has been observed in humans [21]. Both *in vitro* [22] and human studies [23,24] have shown that this incretin secretion highly depends on the origin of the protein and its level of digestion,. It is

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also interesting the fact that a dual bioactive role has been suggested for protein hydrolysates, such as DPP-IV inhibitor and α-glucosidase inhibitor [25], and DPP-IV inhibitor and stimulator of GLP-1 secretion [16,26], highlighting their promising action in the prevention and management of glucose-intolerance pathologies. Therefore, the aim of this study was to obtain bioactive hydrolysates that are able to inhibit DPP-IV activity *in vitro* from proteins of chicken feet using *in vitro* enzymatic treatment. Furthermore, we selected a hydrolysate with high capacity to inhibit DPP-IV activity *in vitro* and evaluated the effect of this hydrolysate on the secretion of GLP-1 in *in vitro* and *ex vivo* models. Finally, we aimed to demonstrate whether this could lead to antihyperglycemic properties in rats.

Material and Methods

Chemicals

Dipeptidyl peptidase IV (from porcine kidney) was purchased from Sigma-Aldrich, Co. (St. Louis, Mo., USA). Gly-Pro-7-amido-4-methylcoumarin hydrobromide (Gly-Pro-AMC) and Pro-p-nitroanilide (Ala-Pro-pNA) were obtained from Bachem AG (Bubendorf, Switzerland). Diprotin A (Ile-Pro-Ile) was from Enzo Life Sciences International (New York, USA) and Vildagliptin was form Axon Medchem (Groningen, The Netherlands). Neutrase® 0.8L and Protamex® were received as a kind gift from Novozyme (Copenhagen, Denmark).

Hydrolysis of chicken feet proteins

Hydrolysates were obtained from chicken feet from chicken feet (*Gallus gallus domesticus)* kindly provided from a local farm (Granja Gaià; La Riera de Gaià, Spain). Chicken feet were washed, triturated, lyophilized and sieved using 2mm pore size sieve to obtain a powder. This powder was subjected to different conditions (of pH, temperature, time and enzyme) to obtain different hydrolysates. First, the powder was diluted in water (at 20 mg/mL) and heated at several temperatures (25, 50 or 100°C) and different pHs (3.0 or 7.5) for 1.5 hours. After this pretreatment, the mixture was hydrolysed at different temperatures (25 or 50°C),pH 7.0 for 2 hours or 24 hours. Also, two different commercial mixes of proteases from *Bacillus amyloliquefaciens* (E.C. 3.4.24), or *Bacillus licheniformis* and *Bacillus amyloliquefaciens* (E.C. 3.4.21.62 and EC 3.4.24.28) so-called Neutrase® and Protamex®, respectively, were used. The different combinations of conditions used can be found in Table 1. In all hydrolysis treatments, the ratio of enzyme:substrate used was 0.4 Anson Units (AU)/g. Final volume of reaction was 5 mL. To stop the hydrolysis reaction, enzymes were inactivated by heating at 80°C for 10 min in a water bath. Solutions were centrifuged at 10000 x *g* for 204 min at 4°C. The supernatants were collected, filtered using 0.45 µm filters and kept at -20°C until further analysis. A control of hydrolysis was prepared too: chicken feet powder diluted in water and pre-treated at 25ºC, pH 7.5, without subsequent hydrolysis (sample called p0H). All conditions were performed by duplicate.

Determination of DPP-IV Inhibitory Activity (% inhibition and IC_{50})

The inhibition assay was performed using 96-well microplates. The DPP-IV enzyme (diluted with 100 mM Tris HCl buffer ph 8.0 to 0.26 mU per well) and 10 µl of of test sample (obtained as described in 2.2) was pre-incubated for 10 min at 37°C. The enzymatic assay started by adding the chromogenic substrate Gly-Pro-pNA (final concentration 0.2 mM). The mixture was measured at 405 nm at 37°C for 30 min in a microplate reader. Diprotin A (Ile-Pro-Ile), a well-known DPP-IV inhibitor, was used as reference inhibitor and positive control. At least three replicates were performed for each determination. DPP-IV inhibition is expressed as a percentage which is the difference of the activity in presence of test peptides versus total activity of the enzyme.

The IC_{50} values were calculated for hydrolysates that achieved the selection criteria: inhibition activity of 80%. To do so, the DPP-IV inhibition assay was performed using a panel of different concentrations of the previously obtained hydrolysates. IC_{50} values expressed in estimated peptide concentration (µg/mL) and volum (µL) were determined by plotting the percentatge of inhibition as a function of the test hydrolysate concentration, using graphPad prism v4.0 for windows.

The nitrogen concentration as an estimation of initial protein content in the hydrolysates was assayed by the Kjeldahl method [27], which determines the total nitrogen content in the sample. The obtained nitrogen content was converted into the protein content by multiplying by 6.25.

Animal studies

Female Wistar rats weighing 180-200g and male Wistar rats weighing 450-500g were obtained from Harlan (Barcelona, Spain). The studies in male groups were performed at the facilities of the Technological Center of Nutrition and Health (www.ctns.cat). Upon arrival, the animals were housed singly in animal quarters at 22ºC with a 12-h light/12-h dark cycle and with free access to food and water. After an adaptation period of one week, the animals were used for the experiments.

Three different experimental models were used to test the acute effects on an oral glucose tolerance test of the hydrolysate selected from *in vitro* studies (named p4H, conditions of hydrolysis are described in Table 1). The dose administered in all the studies was 300 mg of estimated peptide/kg BW, which corresponded to 471.74 mg dried weight of hydrolysate/kg BW. To study the effect of the selected hydrolysate on a **healthy model**, female rats were divided into two groups (n=7): the control group, treated with the vehicle (tap water), and the group receiving p4H, at 300 mg protein/kg of body weight (BW). The same animals were used to study the effect of the p4H on a **diet-induced obesity model**. This model has previously been shown to induce obesity [28]. The rats followed a washing period and then they were fed a cafeteria diet (carrots, bacon, and milk with sugar) plus the standard laboratory chow. After ten weeks, the animals were distributed into a cross-over experimental design, with one week of washing period in between, with two groups (n=7), the control group and the p4H group (300 mg protein/kg BW). Finally, p4H was also tested in a **model of glucose-intolerance due to age** (7-month) [29]. Male Wistar rats were divided into three experimental groups (n=6 rats/group): control group, treated with the vehicle (tap water); p4H group, treated with 300 mg protein/kg BW; and a positive control group, treated with the commercial DPP-IV inhibitor vildagliptin (1 mg /kg BW).

In all the studies, the animals were fasted overnight before experimental treatment. At 9 a.m. on the experimental day, the treatment was administered by intragastric gavage. After 40 minutes, animals underwent an intragastric glucose load (2 g of

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glucose/kg of BW). Tail blood samples were collected into a heparinized capillary tube immediately before and at 15, 30, 60, and 120 minutes after glucose administration. The plasma was immediately separated from the blood by centrifugation (2500 x *g*, 4ºC, 15 min) and stored at -80°C until analysis.

The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) approved all of the procedures (code: 0152S/4655/2015).

Plasma analysis

The glucose plasma levels were assayed using an enzymatic colorimetric kit (Glucose Oxidase-Peroxidase method from QCA, Tarragona, Spain) and DPP-IV plasma activity was measured by a fluorimetric assay as previously described [30].

Cell culture

The STC-1 clonal cell line was received as a kind gift from Dr. B. Wice (Washington University, St. Louis, USA) with the permission of Dr. D. Hanahan (University of California, San Francisco, USA). This enteroendocrine cell line was originated from a double-transgenic mouse tumor [31]. Cells were cultured in DMEM with GlutaMAX containing 4.5 g/L D-glucose, without sodium pyruvate (GIBCO), supplemented with 17.5% fetal bovine serum, 100 U/mL penicillin, and 100 mg/L streptomycin (BioWhittaker) and incubated in a 5% CO_2-humidified atmosphere at 37°C. At least three replicates using different cell passage numbers were performed for each experiment, including at least three wells of each condition in every replicate. Cells were used between passage numbers 30 –50.

***Ex vivo* explants**

Intestinal tissue was obtained from healthy female Wistar rats (n=6). Animals were sacrificed and their intestines were dissected out. To acquire the distal ileum segment, an incision was made in the ileocecal junction and the distal 8 cm of intestine from the ileocecal junction was excised. The ileum was rinsed with ice-cold Hank's balanced salt solution (HBSS; Thermo Fisher Scientific, Madrid, Spain) and was dissected in segments of tissue (0.75 cm^2), which were placed on a 24-well plate and kept on ice until the start of secretion studies.

GLP-1 secretion studies

The stimulation of GLP-1 secretion in STC-1 and *ex vivo* intestinal explants by the hydrolysate selected from *in vitro* DPP-IV inhibitory studies (p4H) was assayed.

STC-1 cells were seeded in 24-well culture plate at a density of 2.0×10^5 cells/well for 2 days until they reached 80-90% confluency. On the day of the experiment, cells were washed twice with HEPES (20 mM HEPES, 140 mM NaCl, 4.5 mM KCl, 1.2 mM $CaCl_2$, and 1.2 mM $MgCl_2$ at pH 7.4), and the selected hydrolysate (p4H) dissolved in HEPES buffer with 10 mM glucose (5 mg/mL) was added to each well. After an incubation period of 2 hours at 37°C, supernatants were collected, centrifuged to remove remaining cells and stored at -80°C until it was used for determination of active GLP-1 concentration.

For the *ex vivo* explant experiments, tissue segments of ileum were placed in prewarmed (37ºC) KRBS buffer (containing 4.5 mM KCl, 138 mM NaCl, 4.2 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 2.6 mM$CaCl_2$, 1.2 mM$MgCl_2$ and 10 mM HEPES (adjusted to pH 7.4 with NaOH)) with 10 mM glucose, 0.1 mM diprotin A and supplemented with 15mg/mL of p4H hydrolysate. Tissue segments were incubated for 1 hour in a humidified incubator at 37°C and 5% CO_2. After the incubation, the solutions were collected, centrifuged to remove remaining cells and stored at -80°C until it was used for determination of active GLP-1 concentration. Tissue viability was checked by an absence of the cytoplasmic marker lactate dehydrogenase (LDH) in the incubated solutions. LDH was analyzed using an LDH kit (QCA; Tarragona, Spain).

Active GLP-1 concentration was measured with a GLP-1 7-37 amide ELISA kit (Millipore; Billerica, MA, USA) following manufacturer's instructions.

Data analyses

The results are expressed as the mean ± SD or mean ± s.e.m. (as indicated in figures and tables). The repeated measurements of glucose were performed using a two-way ANOVA test and significant differences among mean values were determined by post hoc Bonferroni. Differences in the glucose area under the curve (AUC) between groups were determined by using one-way ANOVA, followed by a Bonferroni test. GLP-1 measurement was analyzed using a Student's *t*-test. All calculations were performed using SPSS software (SPSS, Chicago, USA). P-values < 0.05 were considered significant in all cases.

Results

DPP-IV inhibitory activity of chicken feet hydrolysates

Powder obtained from chicken feet was diluted and subjected to different conditions (pretreatment at different temperature and pH followed by hydrolysis treatment with different enzymes, at different temperature and time of incubation) to obtain a panel of different (12) hydrolysates. Table 1 shows the different conditions for each hydrolysate (named p1H to p12H). The DPP-IV inhibitory activity of these hydrolysates was assayed. Table 1 shows that 3 hydrolysates achieved the selected threshold of 80% inhibition. The hydrolysis treatment was essential to obtain hydrolysates with DPP-IV inhibition property since the chicken feet powder that was not subjected to hydrolysis (named p0H, Table 1) did not show inhibitory activity. DPP-IV inhibitory activity was dependent on the conditions of pretreatment and hydrolysis treatment. The results show that the treatment of chicken feet in all tested hydrolysis conditions, leads to samples with DPP-IV inhibitory capacity. When the treatment is performed after a pretreatment in basal conditions, consisting on an incubation for 1.5 hours at 25°C and pH 7.5, DPP-IV inhibitory capacity achieves the threshold of around 40% (p1H, p5H, p9H). Modification of the pretreatment conditions by reducing the pH to 3 and increasing the temperature (to 50°C or 100°C) (samples p4H, p8H, p12H in Table 1) leads to samples with the highest DPP-IV inhibitory activity (80-100%).

Effects of chicken feet hydrolysate (p4H) on plasma glucose

The effect of an acute administration of the selected hydrolysate named p4H was tested in two different models of rats with glucose intolerance: diet-induced and age-induced.

In a cafeteria-induced obese rat model, the administration of p4H (at 300 mg/kg BW) together with a glucose load reduced the

peak of glucose and tended to normalize the glucose values at 120 min (Figure 1.A). The glucose AUC value after the OGTT was lower in the p4H-treated animals than in the control cafeteria group (Figure 1.B). Also in aged rats, which had glucose intolerance (in non-treated animals, glucose levels at 120 min did not return to initial levels) a p4H administration ameliorated the plasma glucose profile similarly to what the positive control vildagliptin did (Figure 2).

The effects of the hydrolysate in rats with normal glycemia were also tested. Actually, this was performed in the same group of rats that were subjected to a cafeteria diet, but previously to the cafeteria treatment. In this case, the p4H had no significant effect (Δ Glucose AUC (arbitrary units): 2658.50 ± 902.25 and 3201.28 ± 504.41, control and p4H-treated group, respectively).

Stimulation of GLP-1 release by Chicken feet hydrolysate

Finally, we tested the capacity of the selected hydrolysate p4H to stimulate GLP-1 release *in vitro* and *ex vivo*. In STC-1 enteroendocrine cells, treatment with 5 mg p4H /mL for 2 hours led to a strong (7-fold) increase in GLP-1 levels in the media compared to controls (Figure 3. A). Similarly, GLP-1 secretion from ileum tissue segments was stimulated in response to 15 mg p4H /mL (Figure 3.B).

Discussion

In this paper, we focused on finding chicken feet hydrolysates that act as DPP-IV inhibitors and inductors of the endogenous secretion of GLP-1, which altogether could be a useful strategy against Type 2 diabetes.

We define for the first time that whole chicken feet hydrolysates are able to inhibit DPP-IV activity. Other food-derived proteins have previously been shown as sources of DPP-IV inhibitory peptides, such as whey [32], milk [33], salmon skin [34] and quinoa [35]. In this paper we performed a screening in which chicken feet were subjected to different hydrolysis conditions, and DPP-IV inhibitory activity was then assayed. We found that not all the hydrolysis treatments led to the same results, indicating the importance of the hydrolysis conditions in the hydrolysates behaviour. Three protocols leading to hydrolysates with high DPP-IV inhibitory activity were found. The pretreatment consisting on an incubation for 1.5 hours at 50°C or 100°C and pH 3, previously to the addition of the enzyme,was crucial to obtain hydrolysates with high DPP-IV inhibitory capacity. In fact, the three hydrolysates that showed a DPP-IV inhibitory activity higher than 80% presented similar pharmacological potency, since IC_{50} values expressed as microliter are similar (around 4 µL), and also similar pharmacological specificity, shown by similar IC_{50} values expressed as micrograms of estimated peptide per millilitre (around 300 µg/mL). The IC_{50} of these hydrolysates is 10-fold lower than that found in a previous study which used collagen obtained from chicken-feet and hydrolysed using *Streptomyces* collagenase (45°C, pH 7.5, 17 h) [36]. Thus we here show for the first time that the use of the whole chicken feet, a byproduct of food industry, without requirement of a previous collagen purification, and hydrolysed under the appropriate conditions (here defined) can lead to samples with stronger DPP-IV inhibitory potency.

The obtained IC_{50} values are within the range of these observed for other food hydrolysates [11,37]**.** A lower potency of the food hydrolysates compared to purified peptides has been found in other studies, suggesting that the concentration of bioactive peptide in the hydrolysate is low [33]. . Thus, despite a further identification of the bioactive peptides is of interest, we here present important clues to prepare hydrolysates from chicken feet for its use as antihyperglycemics.

We next selected one of the chicken feet hydrolysates, named p4H, to prove its bioactivity *in vivo*. It must be taken into account that a high DPP-IV inhibitory activity *in vitro* could not involve an antihyperglycemic effect *in vivo*. During the gastrointestinal digestion, the bioactive peptides contained in the samples could be hydrolysed and transformed into inactive peptides. For this reason, it is essential to validate the hydrolysate bioactivity *in vivo*. We tested the p4H effects at reducing glycaemia after an oral glucose tolerance test in a model of rats fed a cafeteria diet. Our results show that the p4H treatment led to a lower glucose peak and a lower glucose curve, with a tendency to reach the glucose levels of the standard-fed animals. Instead, in healthy rats p4H did not modify the AUC of the glucose load. This could be related to the fact that in the cafeteria-fed rats the glucose peak was greater (an increase of around 80 mg/dL at 15 min) than when animals are subjected to normal feeding (glucose increase of around 50 mg/dL), due to their disorder in glucose homeostasis. This disorder induced by the cafeteria diet is evident at 120 min after the OGTT, when the glycaemia of cafeteria-fed animals did not return to basal levels, while under normal feeding the rats showed normal OGTT curves. The p4H hydrolysate effects was also tested in another model of glucose-intolerance, which are aged rats. In this model, an acute treatment with p4H also lowered the plasmatic glucose curve after an OGTT. We therefore show that an acute dose of food hydrolysate influences glucose homeostasis in rat models with glucose intolerance. Only a few potential DPP-IV inhibitor-peptides have previously been tested *in vivo* for their glucose lowering capacity, and this was performed in healthy models. Uchida et al. tested a ß-lactoglobulin hydrolysate in mice and observed that glucose levels were reduced at 15 min after an OGTT. It worth mentioning that the glucose dose administered was 10 g/kg BW, much higher than the one we have tested (2 g/kg BW) and therefore led to a great (around 3-fold) increase in glucose peak at this time point [14], reinforcing that the effects are dependent on the amount of circulating glucose. This might be linked to the modest potency of the food hydrolysates compared to commercial purified DPP-IV inhibitors, which we and others [14] observed. Interestingly, in aged control animals, the comparison of p4H-treated and vildagliptin-treated animals showed a different glucose profile: the p4H was not as effective as vildagliptin at reducing the glucose peak but normalized its glycemia at 120 min in a similar way. These results suggest that p4H might not act exactly through the same mechanism than vildagliptin to ameliorate hyperglycemia.

It must be stated that we measured DPP-IV activity in plasma from the tail of the rats and found no effect of p4H in any of the animal experiments (results not shown). However, it has been shown that most of GLP-1 degradation occurs immediately at the site of secretion, and only 10-15% active GLP-1 levels remain in the systemic circulation [2,38]**.** Although various hydrolysates have been reported to reduce plasma DPP-IV activity after chronic treatments [39–41], few studies have shown such reduction in acute treatments. Actually, whey protein has been reported to attenuate hyperglycaemia in part through inhibition of DPP-IV activity in the small intestinal tissue, but not in orbital vein [42]. It has also been shown that ileal administration

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of Zein and rice protein hydrolysates decreased plasma DPP-IV activity in ileal vein [16,26]. And in fact, none of the other papers describing *in vivo* antihyperglycaemic effects of protein hydrolysates show values of DPP-IV activity in peripheral blood [14,15]. Therefore, it cannot be excluded that, despite the lack of DPP-IV inhibition in peripheral blood, p4H leads to an amelioration of glucose profile due to the inhibition of local intestinal DPP-IV.

It has previously been shown that Zein and rice protein hydrolysates have antihyperglycaemic effects through a dual mechanism of action, both inhibiting DPP-IV activity as well as increasing GLP-1 release [16,26]. In the study of Mochida et al.[16], meat hydrolysate (acquired from Sigma, no description of the meat origin) was also tested and found to induce intestinal GLP-1 secretion, although it did not show the dual effect. Other protein hydrolysates have been tested for its potential role as GLP-1 release stimulators *in vivo, in situ* and *in vitro* [17,21,22,43]. We reproduced this approach and tested whether our chicken feet hydrolysate p4H could also modulate GLP-1 release. We used *in vitro* and *ex vivo* models, and our results show a strong significant increase in GLP-1 levels after p4H treatment in both models. The form in which the hydrolysate might reach the enteroendocrine L-cells *in vivo* might differ from that used in our models. However, our results suggest that stimulation of GLP-1 release might also be a mechanism to reduce glycaemia in animals. This additional mechanism together with the DPP-IV inhibition could help to explain the above-mentioned differences found between the DPP-IV inhibitor vildagliptin and the p4H in the aged-rats experiment. Further experiments will be required to confirm this hypothesis.

Conclusions

In conclusion, we here report that chicken feet hydrolysates are a source of DPP-IV inhibitory peptides, and define the hydrolysis conditions to obtain a higher inhibitory potency. Furthermore, we show that a chicken feet hydrolysate obtained by Neutrase® treatment reduced glycaemia in glucose-intolerant rats and that this might be in part through its DPP-IV inhibitory activity. In addition, a stimulation of endogenous GLP-1 secretion might also be involved in the antihyperglycaemic action of this hydrolysate.

Conflicts of interest

There are no conflicts to declare.

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Tables

Table 1. Chicken feet hydrolysates with dipeptidyl peptidase IV inhibitory activity.

Hydrolyzate	Pre-treatment (1.5 h)		Treatment (pH 7)			DPP-IV Inhibition (%)
	Temp (°C)	pH	Enzyme	Temp (°C)	Time (h)	
p0H	25	7.5	No			0
p1H	25	7.5	Neutrase®	25	24	48.39
p2H	50	7.5	Neutrase®	25	24	44.40
p3H	25	3	Neutrase®	25	24	60.48
p4H	**50**	**3**	**Neutrase®**	**25**	**24**	**83.22**
p5H	25	7.5	Neutrase®	50	24	40.22
p6H	100	7.5	Neutrase®	50	24	77.19
p7H	25	3	Neutrase®	50	24	55.24
p8H	**100**	**3**	**Neutrase®**	**50**	**24**	**100**
p9H	25	7.5	Protamex®	50	2	44.05
p10H	100	7.5	Protamex®	50	2	60.49
p11H	25	3	Protamex®	50	2	53.30
p12H	**100**	**3**	**Protamex®**	**50**	**2**	**93.30**

The IC_{50} of the three selected hydrolysates was calculated and expressed in volume (μL) and estimated peptide concentration (μg/mL). The IC_{50} given in volume measures the hydrolyzate pharmacological potency since this value signifies the volume required to inhibit the enzyme by 50% under the assay condition. The expression of IC_{50} in protein concentration means the specificity of peptide pool mixture against DPP-IV, hence it is an indicator of the pharmacological specificity. All of them showed similar values between 4.03 and 4.82 μL or 297.4 and 302.9 μg estimated peptide/mL (Table 2). The hydrolysate named p4H (pretreatment 50º, pH3; hydrolysis with Neutrase, 25º, pH7, 24h) was selected for posterior experiments, since the temperature of pre-treatment (50º) and hydrolysis (25º) were lower than in the other two hydrolysates (100 and 50 for pretreatment and treatment respectively), and this could imply an industrial production less expensive

Table 2. Comparison between estimated peptide content and DPP-IV Inhibitory Activity of hydrolysates of chicken feet produced by enzymatic treatments.

hydrolysate	Estimated peptide content (mg/mL) [1]	IC_{50} (µL)[2]	IC_{50} (µg/mL)[2]
p4H	6.24	4.82 ± 0.2	302.9 ± 9.8
p8H	6.83	4.42 ± 0.1	297.4 ± 4.6
p12H	6.62	4.45 ± 0.2	300.1 ± 10.2

[1] Nitrogen compounds content was measured by Kjeldahl method. [2] IC_{50} values are reported as the mean from duplicate assays ± SD.

Figure 1

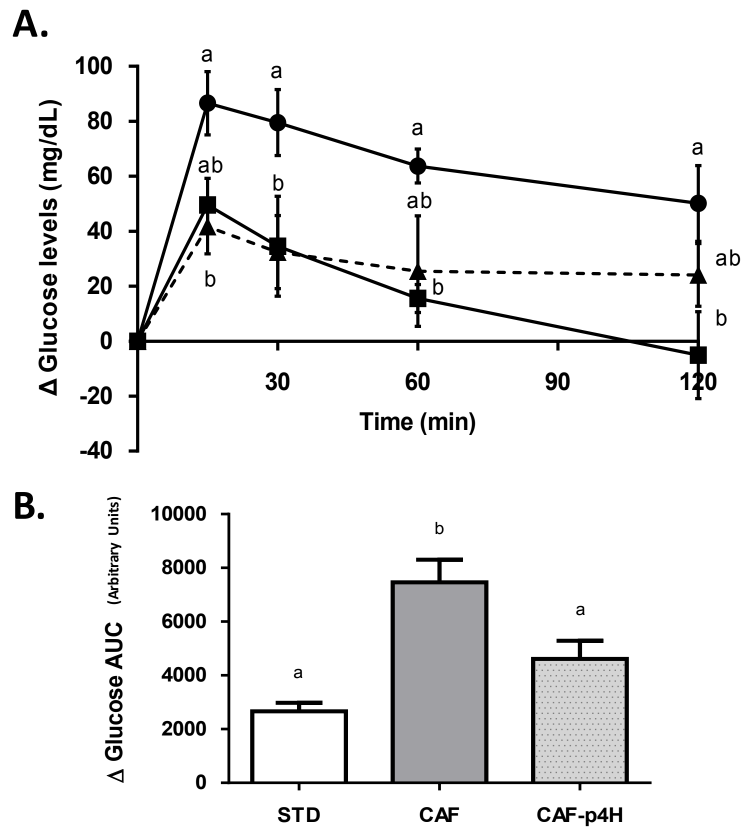
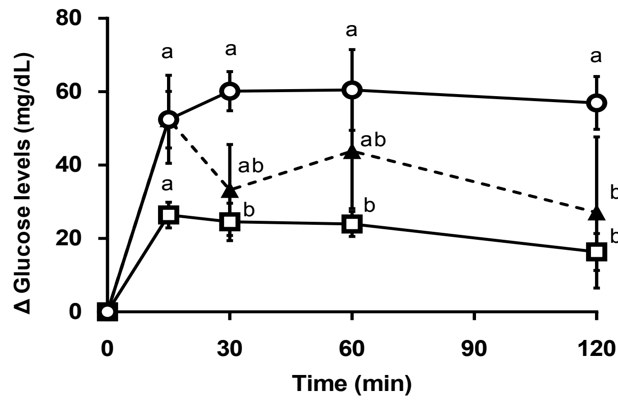


Figure 2

A.



B.

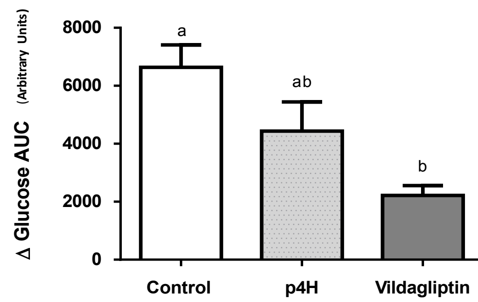


Figure 3