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Peptides from chicken processing by-product inhibit DPP-IV and promote cellular glucose uptake: potential ingredients for T2D management

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Abstract

Inhibition of dipeptidyl peptidase IV (DPP-IV) and stimulation of muscle glucose uptake are two of the key strategies for management of type-2-diabetes (T2D). In the present study, four protein hydrolysates generated by enzymatic hydrolysis of chicken by-product, i.e., mechanical chicken deboning residue, were evaluated for their DPP-IV inhibitory activity as well as their effect on glucose uptake by skeletal muscle cells. The DPP-IV inhibitory assay was performed in two concentrations (1000 µg/mL and 10 µg/mL) for the crude chicken protein hydrolysates. The hydrolysate with the highest DPP-IV inhibition was selected for preparative-scale fractionation using size-exclusion chromatography (SEC). The SEC fractions were tested for DPP-IV inhibitory activity as well as their effect on glucose uptake and metabolic activity of skeletal muscle cells. The muscle cells were treated with the SEC fractions and glucose uptake was measured based on luminescence detection of 2-deoxyglucose-6-phosphate (2DG6P). A fraction with peptides in the lower molecular weight range was shown to promote glucose uptake and to inhibit DPP-IV. Further chromatographic fractionation followed by inhibition assaying of the most potent SEC fraction led to isolation of five refined peptide fractions with more than 80 % DPP-IV inhibition, which were subsequently analyzed with LC-HRMS/MS. This led to identification of 14 peptides as potential DPP-IV inhibitors from protein hydrolysates of mechanical chicken deboning residue.

Keywords: Dipeptidyl peptidase IV, chicken protein hydrolysates, cellular glucose uptake, antidiabetic, bioactive peptides.

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1. Introduction

Type 2 diabetes (T2D) is a metabolic syndrome characterized by chronic hyperglycemia and is related to several complications such as nephropathy, retinopathy and neuropathy.[1] Hyperglycemia is generally provoked by insufficient secretion of insulin by the pancreatic β-cells or inability of the cells to respond to insulin.[2] About two-thirds of the insulin secretion is due to the action of the incretin hormones glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP).[3] These hormones are downregulated by dipeptidyl peptidase IV (DPP-IV), a prolyl peptidase that rapidly cleaves proteins and peptides after a proline amino acid residue. The half-life of the incretin hormones is short (less than two minutes), and their degradation by DPP-IV will consequentially have a negative impact on insulin secretion from pancreatic beta cells. Inhibitors of DPP-IV have therefore become promising therapeutics for the management of T2D as an alternative to conventional therapies targeting the decrease of hepatic glucose production (biguanides, i.e. metformin), PPAR-γ agonists (i.e. thiazolidinediones), inhibitors of carbohydrases like α-amylase and α-glucosidase (i.e. acarbose, voglibose).[4] Currently, there are few DPP-IV inhibitors in clinical use, such as sitagliptin, vildagliptin and saxagliptin. Despite efficient hypoglycemic effect, these drugs are expensive, and their long-term safety remains unestablished.[5] There is, therefore, a need for alternative sources of DPP-IV inhibitors in the form of functional food or nutraceuticals.

An alternative therapeutic approach for management of hyperglycemia is increasing utilization of glucose by the peripheral tissues and consequently lowering hepatic glucose output. It is well documented that increasing glucose uptake in muscle cells, in the absence of insulin, can be achieved through exercise.[6] However, some studies suggest that peptides derived from food protein can also promote glucose uptake. For example, branched-chain amino acid containing

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dipeptides derived from whey have been shown to promote glucose uptake in both L6 myotubes and isolated skeletal muscles.[7,8]

Over the past decade, a significant number of scientific studies have emphasized the potential health-promoting effect, including antidiabetic properties, of dietary protein hydrolysates recovered from a wide range of by-products.[9-13] This has high industrial relevance, since there is a continued search for feasible applications of food processing by-products that can lead to increased profits for the producers. Protein hydrolysates from by-products of both plant- and animal-based food processing have been shown to exhibit antidiabetic activities through different mechanisms, including α-glucosidase and DPP-IV inhibition.[14-16] There are a number of successful developments of bioactive peptides from diary by-products as food supplements. However, similar achievements are yet to be accomplished in valorization of meat and poultry by-products. This is largely due to the high degree of biochemical complexities of meat and poultry by-products and the resulting peptide mixture generated by, in most cases, non-specific enzymatic digestion. Therefore, most of the studies on such complex by-products (for example, poultry by-products) are limited to evaluation of the crude hydrolysates and the observed activities are seldom ascribed to particular peptides or set of peptides.

Among the modern analytical approaches for discovery of bioactive constituents in complex mixtures is the use of chromatography-coupled bioassays where eluents of a separation are directed to bio-screenings. Results of the bio-screenings, known as biochromatograms, aligned with the chemical profiles from the chromatographic detector will provide an excellent tool to unequivocally pinpoint bioactive constituents or fractions of a complex matrix. This approach, in combination with mass spectrometry and nuclear magnetic resonance spectroscopy, have been successfully used to identify bioactive constituents from plant-based crude extracts.[17-20] In the current study, we have applied such a chromatography-coupled bio-screening strategy to characterize peptides recovered from protein hydrolysates of a chicken by-product. In addition

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to DPP-IV inhibition, the crude protein hydrolysates and the different peptide fractions were evaluated for effect on cellular glucose uptake. The sequences of DPP-IV inhibitory peptides from a chicken by-product as well as their effect on cellular glucose uptake is reported here for the first time.

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2. Materials and Methods

2.1. Sample material and chemicals

Mechanical chicken deboning residue (MCDR) was provided by a Norwegian slaughterhouse (Nortura, Hærland, Norway). Corolase 2TS was purchased from AB enzymes (Darmstadt, Germany). DPP-IV from porcine kidney (EC 3.4.14.5) was purchased from Merck (Merck, Darmstadt, Germany). Protease from *Aspargillus oryzae* (Flavourzyme), insulin, Gly-pro-*p*-nitroanilide (GPPN), Tris, diprotin A, HPLC-grade acetonitrile, formic acid, trifluoroacetic acid (TFA) and molecular weight standards (bovine serum albumin, albumin from chicken egg white, carbonic anhydrase from bovine erythrocytes, lysozyme, cytochrome C from bovine heart, aprotinin from bovine lung, insulin chain B oxidized from bovine pancreas, renin substrate tetradecapeptide porcine, angiotensin II human, bradykinin fragment 1-7, [DAla2]-leucine encephalin and Val-Tyr-Val) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amphotericin, Dulbecco's Modified Eagle Medium (DMEM), 0.05% trypsin/ EDTA, fetal bovine serum (FBS) and penicillin/streptomycin solution 10 000 units/mL (P/S) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ultroser G serum substitute was purchased from Pall Biosepra (Cergy-Saint-Christophe, France).

2.2. Production of enzymatic protein hydrolysates

The production of hydrolysates was performed according to a previously published protocol.[21] In short, 500 g of MCDR was homogenized using a food processor and was mixed with 1 L of water in a Reactor-Ready™ jacketed reaction vessel (Radleys, Saffron Walden, Essex, United Kingdom). Water circulating through the jacket of the reactor was heated to 50°C and delivered by a JULABO circulator pump (Julabo, Seelbach, Germany). After slowly mixing the MCDR until the temperature reaches 50 °C, 7.5 mL of enzyme was added to start the reaction. A total of four hydrolysates were produced: an 80-min hydrolysate with flavourzyme (RF80), a 240-

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min hydrolysate with flavourzyme (RF240), a 80-min hydrolysate with corolase (RC80) and a 240-min hydrolysate with corolase (RC240). After the specific hydrolysis time (80 minute or 240 minute), the enzymes were inactivated by heating in a water bath at 95 °C for 15 min. Contents of the reaction mixture were subsequently centrifuged for 15 min at 4600g and 4 °C to afford a water-phase supernatant, a fat-phase and solid residue. The water phase was lyophilized to afford a light yellow-colored powder of protein hydrolysates.

2.3. Preparative size exclusion chromatography

Chromatographic separation of the protein hydrolysate RC80 was performed with a Dionex Ultimate 3000 series instrument (Thermo Scientific, Waltham, MA, USA) equipped with a quaternary pump, an autosampler, an RS variate wavelength UV-Vis detector, and an automated fraction collector. Separation was carried out at 25 °C using a Phenomenex BioSpe-SEC-s2000 column, 300 × 7.8 mm i.d., 5 μm particle size, 145 Å pore size (Phenomenex, Torrance, CA, USA) and mobile phase consisting of 0.1 M phosphate buffer, pH 6.8. Isocratic elution was carried out using a flow rate of 4 mL/min for 40 min and monitored at 214 nm. An injection volume of 1 mL of aqueous solution of RC80 (100 mg/mL) was used and eight fractions (F1-F8) were collected from 9 to 29 minutes. Collected fractions were lyophilized and stored at 4 °C before further use. Chromatographic runs were controlled using Chromeleon software version 7.2 SR4 (Thermo Scientific, Waltham, MA, USA). For the molecular weight standards an injection solution 2 mg/mL was prepared in water. For each standard, 15 μL was injected and separation was performed with the same condition as for the samples above.

2.4. Preparative-scale reversed-phase chromatography

Orthogonal chromatographic separation of fraction F5 was performed using a Dionex Ultimate 3000 series instrument (Thermo Scientific, Waltham, MA, USA) equipped with a quaternary pump, an autosampler, an RS variate wavelength UV-Vis detector, and an automated fraction

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collector. 1 mL of aqueous solution of fraction F5 (100 mg/mL) was separated at 25 °C using a Thermo Betasil C_{18} column, 250 × 10 mm i.d., 10 µm particle size (Thermo Scientific, Waltham, MS, USA). Mobile phase consisted of water (solvent A) and acetonitrile (solvent B), both acidified with 0.05% of TFA, and the flow rate was kept at 4 mL/min. A gradient elution was carried out as follows: 0 min, 0% B; 10 min, 0% B; 45 min, 40% B; 50 min, 100% B; 60 min, 100% B. The separation was monitored at 214 nm, and 18 fractions were collected from 13 to 40 minutes. Fractions from a single separation were subsequently lyophilized and used for DPP-IV inhibition. Subsequently, four similar separations were performed and collected fractions were pooled and used for LC-MS/MS analysis. Chromatographic separation was controlled using Chromeleon software version 7.2 SR4 (Thermo Scientific, Waltham, MA, USA).

2.5. DPP-IV inhibition assays

2.5.1. Screening of crude protein hydrolysates and fractions

The crude hydrolysates were assayed using final concentrations 10 µg/mL or 1000 µg/mL whereas the SEC fractions were assayed using a final concentration of 1000 µg/mL. Test samples of the lyophilized reversed phase fractions were prepared by directly dissolving the lyophilized fractions in 20 µL of assay buffer. The DPP-IV inhibition assay was performed according to Al-Masri et al. (2009) with slight modifications.[22] In short, experiments were performed in triplicate in 96-well microplates with a final volume of 100 µL. 20 µL test sample, 22.5 µL of Tris-HCl buffer pH 7.5, and 7.5 µL of DPP-IV enzyme solution in Tris-HCl buffer pH 7.5 (0.05 U/mL final concentration) were added to each well. The mixture was incubated for 10 minutes at 37 °C, whereafter 50 µL of GPPN (0.2 mM in Tris-HCl, pH 7.5) was added to the mixture. The absorbance was subsequently measured at 405 nm every 1 min for 30 min, using a Synergy H1 hybrid multi-mode microplate reader (Biotek, Winooski, VT, USA).

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Diprotin A was used as a positive control. The percentage of DPP-IV inhibition was calculated using the following formula:

$$\%\ Inhibition = 1 - \left(\frac{Slope_{sample}}{Slope_{control}}\right) \times 100$$

2.5.2. Determination of DPP-IV IC_{50} values for RC80 and fraction F5

The DPP-IV IC_{50} values of RC80 and the active fraction F5 were determined using the standard methods described in Section 2.5.1. The percentage of inhibition of DPP-IV was calculated as mean ± standard deviation in Microsoft Excel using the above-described formula. The results were thereafter exported and used to assess the dose-response curves and IC_{50} values in GraphPad Prism, version 7.04 software (La Jolla, CA, USA). Data were fitted into the equation:

$$f\ (x) = \min + \frac{max - min}{1 + (\frac{x}{IC_{50}})^{slope}}$$

where min is the background, max-min is the y-range, x is the concentration and slope is the Hill slope.

2.6. In vitro primary skeletal muscle cells

2.6.1. Cell seeding and treatment

Bovine primary skeletal muscle satellite cells were isolated as previously described.[23] Animals of the same age (young animals), gender (bulls) and breed (Norwegian Red) were used for the muscle cell isolation. In brief, small muscle pieces of ~ 1 g were digested for 1h with 70 rpm shaking in 10 mL DMEM with 0.72 mg/ml collagenase, 10 000 units/mL P/S and 250 µg/mL amphotericin B at 37°C. The muscle cells were subsequently dissociated from surrounding tissue by three cycle treatments (of 25 min each) with 0.05% trypsin/EDTA. 10% FBS were

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added after each treatment to inactivate trypsin and harvested cells were pooled. For removal of fast-adhering fibroblasts from the primary muscle cell cultures, the cells were placed in un-coated cell flasks for 1 h at 37 °C which allowed the fibroblasts to adhere to the plastic. The non-adhering primary muscle cells were then collected in low glucose DMEM GlutaMAX™ containing 2% FBS, 2% Ultroser G, P/S (10 000 units/mL) and amphotericin B (250 µg/mL), seeded out (3000 cells/well) in to a 96 well plate and were grown for four days until 70-80 % confluence. Subsequently, the cells were placed in differentiation medium (i.e., DMEM containing 2% FBS, P/S (10 000 units/mL) and amphotericin B (250 µg/mL) and 25 pmol insulin) for three days to induce myogenesis. The differentiated primary bovine muscle cells were then used to measure glucose uptake and cell viability (ATP production).

2.6.2. Cell glucose uptake and metabolic activity measurements

Both glucose uptake and metabolic activity (viability) measurements were performed in triplicates. One day prior to treatment with the hydrolysate fractions, the differentiated primary bovine muscle cells were starved with serum free medium for 24 hours. For the glucose uptake study, the cells were treated with 100 µL of 1 mg/mL solution of the eight SEC fractions (F1-F8) for 1 hr at 37 °C and with 5% CO_2. Glucose uptake, after the treatment, was measured based on luminescence detection of 2-deoxyglucose-6-phosphate (2DG6P). The measurements were performed using a Glucose Uptake-Glo™ Assay kit (Cat# J1341; Promega, Madison, WI, US). Incubation with insulin (1 mM) was used as a positive control and percentage glucose uptake was calculated relative to untreated cultures (100 % glucose uptake). As a negative control, glucose uptake was calculated for cells without addition of 2-deoxyglucose (-2DG) and with addition of stop buffer prior to 2DG (stopped). Effect of the eight SEC factions (at a final concentration of 500 µg/mL) on metabolic activity of the cells in the culture was measured based on quantification of the ATP present. The differentiated cells were treated with 100 µL

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of 1 mg/mL solution of the eight SEC fractions (F1-F8) for 1 hr at 37 °C and with 5% CO_2 and relative quantification of ATP production was determined using a Promega CellTiter-Glo® luminescent cell viability assay kit (Cat# G9241; Promega, Madison, WI, US). Percentage ATP production for the cultures treated with the SEC fractions was calculated relative to untreated cultures (100 % ATP production). Luminescence was measured using a Synergi H1 hybrid multi-mode reader (BioTek Instruments, Inc., Winooski, VT, USA)

2.7. HPLC-HRMS analysis

An injection solution of each sample was prepared by dissolving the freeze-dried fraction in 50 % methanol. High-performance liquid chromatography-high-resolution mass spectrometry (HPLC-HRMS) analyses of fractions F5-4, F5-5, F5-13, F5-14, and F5-16 were performed on a Agilent 1260 chromatograph consisting of a G1322A degasser, a G1311A quaternary pump, a G1316A thermostatted column compartment, and a G1315A photodiode-array detector (Santa Clara, CA, USA) hyphenated with a Bruker micrOTOF-Q II mass spectrometer equipped with an electrospray ionization (ESI) interface and controlled by Bruker Hystar software version 3.2 (Bruker Daltonik, Bremen, Germany). Separation of freeze-dried samples re-dissolved in 50 % methanol were performed on a reversed-phase Phenomenex Luna® Omega Polar C_{18} column, 250 × 4.6 mm, 5 mm particles, 100 Å pore size (Phenomenex, Torrance, CA, USA) using an injection volume of 10 µL. The flow rate was maintained at 0.5 mL/min, using the following gradient elution profile of mobile phase A (water/acetonitrile 95:5 v/v) and mobile phase B (water/acetonitrile, 5:95 v/v), both acidified with 0.1% formic acid: 0 min, 0% B; 5 min, 0% B; 25 min, 100% B; 35 min, 100% B; 37 min, 0% B. Automated MS/MS spectra were acquired in positive ion mode, using a drying temperature of 200 °C, a nebulizer pressure of 2.0 bar, and a drying gas flow of 7 L/min. For smaller molecular weight peptides (less than 500 Dalton) identification was performed manually by studying the fragmentation patterns. For peptides of 8 amino acid residues or more, database-assisted identification was performed using MaxQuant

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software version 1.6.2.3.[24] Raw LC-HRMS/MS data was searched against unspecific digest of *Gallus gallus* (Chicken) proteins (UniProtKB database).

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3. Results and discussion

Four crude protein hydrolysates produced from mechanical chicken deboning residues (hydrolyzed for 80 or 240 minutes) using flavourzyme (RF80 and RF240) and corolase (RC80 and RC240) as catalytic proteases were assessed for their DPP-IV inhibition. The hydrolysate with the highest activity was fractionated using SEC, and these fractions were assessed for their DPP-IV inhibitory activity as well as their effect on glucose uptake. This led to isolation of the most bioactive fraction F5. Following further reversed-phase chromatographic fractionation of F5, DPP-IV inhibition assaying, and LC-HRMS/MS analysis, the most promising hypoglycemic peptides were identified. A summary of the work flow is presented in **Figure 1**.

3.1. DPP-IV inhibition of the chicken protein hydrolysates

A preliminary assessment for DPP-IV inhibition, at two different concentrations showed that the crude hydrolysates had 45% to 60% inhibition at 1 mg/mL (**Table 1**). Recent studies have shown that by-products from macroalgae, fish, whey protein, and chicken egg proteins have potential antidiabetic effects due to DPP-IV inhibition.[15, 25] However, to the best of our knowledge, this is the first report of chicken byproduct hydrolysates as source of DPP-IV inhibitors. One of the trends observed from the preliminary screening was a decrease in DPP-IV inhibition with an increase of the hydrolysis time from 80 to 240 minutes. This could be a result of breakdown of the bioactive peptides into single amino acids. The most active hydrolysate (RC80) from the screening was found to have an IC_{50} value of 0.919 mg/mL (**Figure S1**). This IC_{50} value is comparable to previously reported DPP-IV inhibition by protein hydrolysates from different sources.[26,27] Thus, the mechanical chicken deboning residue hydrolyzed with corolase for 80 minutes (RC80) was chosen for an in-depth analysis and identification of its bioactive constituents.

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3.2. SEC-coupled DPP-IV inhibition

Raw protein hydrolysates contain molecules ranging from unhydrolyzed large proteins to simple amino acids. Therefore, a targeted study of bioactive constituents usually requires a fractionation or a filtration procedure. In the current study, the most active protein hydrolysate (RC80) was subjected to SEC fractionation, where eight fractions (F1-F8) were automatically collected (**Figure 2A**). The overall chromatogram of RC80 showed abundance of constituents in lower molecular weight range (retention time below 19 min). After measuring the DPP-IV inhibition for the eight fractions, the bioactivity profile (**Figure 2B**) was plotted under and was correlated to chromatographic trace (**Figure 2A**). This provided a tool to identify and guide a targeted isolation the promising bioactive peptide fraction. The most active fraction (F5) was eluted from 19-21 min and showed 54 % inhibition of DPP-IV (**Figure 2B**). This promising fraction (F5) was therefore subjected to dose-dependent DPP-IV assessment and was found to have an IC_{50} of 0.155 mg/mL. This showed that this fraction possesses approximately six-fold higher DPP-IV inhibitory activity than that of the raw protein hydrolysate (RC80; IC_{50} = 0.919 mg/mL). The increased DPP-IV inhibition of fraction F5 is a result of the targeted fractionation of the most bioactive peptides assisted by the bioactivity profile (**Figure 2B).**

3.3. Effect of peptide fractions on cellular glucose uptake and metabolically active cells

The eight SEC fractions (F1-F8) were studied for their effect on glucose uptake by skeletal muscle cells. The results showed that three of the eight fractions (F-5, F-6 and F-7) induced cellular glucose uptake (**Figure 3A**). Particularly, fraction F5 resulted in increased glucose uptake by 41.6 % at a concentration of 1 mg/mL. Recent studies have reported positive effects of peptides from soybeans and flaxseeds protein hydrolysates on glucose uptake.[28] Moreover, it has been reported that an improved glucose uptake effect might be related to specific low

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molecular weight peptides (between 300-400 kDa).[28,29] To the best of our knowledge, this is the first study reporting the positive effect of peptides from chicken hydrolysates on glucose uptake. In addition to the glucose uptake, the fractions F1-F8 were tested for the effect on metabolic activity of the cells by measuring the ATP present in the cell cultures. In particular fraction F5 decreased ATP production in the cell culture (**Figure 3B**), but also fractions F3, F4 and F6 seems to decrease ATP production - albeit to a lower extent. One possible explanation to this result might be activation of AMP-activated protein kinase (AMPK). AMPK is suggested to be one of the targets of major antidiabetic drugs, such as thiazolidinediones and the biguanides, as well as insulin sensitizing adipokines, although the mechanism seems to be indirect.[30,31] Several studies suggest that AMPK plays an important role during muscle glucose uptake during pharmacological stimuli which is consistent with the observed lowest ATP production for cells stimulated with F5 (a fraction correlated with highest glucose uptake).[32] AMPK function as a sensor of intracellular energy, and pharmacological activation of AMPK has been shown to promote glucose transport.[33] At the same time, AMPK is activated by increased cellular level of AMP:ATP and ADP:ATP ratios, which could explain the reduced ATP levels observed in the cell culture stimulated with F5.[32,34] The high complexity of the mechanisms and receptors involved in cellular glucose uptake, in particular the AMPK pathway upon peptide treatment, requires further investigations in order to establish the most probable pathways responsible for the activity of the tested fractions.

3.4. Reversed-phase chromatography-coupled DPP-IV inhibition

Despite preliminary SEC fractionation, the protein hydrolysate fractions are still typically associated with a high degree of complexity. One of the successful analytical approaches to resolve constituents of complex biological matrices is sequential orthogonal chromatographic separations.[35] In this study, a reversed-phase chromatography separation, orthogonal to the preceding SEC fractionation, was performed on the bioactive fraction F5 and a total of 18

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reversed-phase fractions were automatically collected from 13 to 40 minutes (**Figure 4A**). These fractions were subsequently evaluated for their DPP-IV inhibitory activity. The bioactivity of the different fractions was plotted against the retention time leading to a semi-high-resolution DPP-IV inhibition profiling (**Figure 4B**). The majority of the fractions were shown to have moderate to high activity, and fractions F5-4, F5-5, F5-13, F5-14 and F5-16, showed DPP-IV inhibitory activity above 80% (highlighted in **Figure 4A**). The highest activities were observed for both early eluting peptides (F5-4 and F5-5) as well as the three late eluting peptides fractions (F5-13, F5-14 and F5-16). This is in contrast to a previous study, which has proposed a link between retention time of peptides in a reversed-phase column and DPP-IV inhibitory activity.[15] The five most active fractions were subjected to LC-HRMS/MS analyses for identification of bioactive peptides.

3.5. Identification of bioactive peptides

The base peak chromatograms of fractions F5-4, F5-5, F5-13, F5-14 and F5-16 are presented in **Figure 5**. The complete list of the 19 major constituents from these five most bioactive fractions with retention time, MS/MS results and identified peptides is presented in Table 2. In addition, all the MS and MS/MS spectra are provided in supplementary information (**Figure S2-S19**). Of the 19 major constituents, a very high degree of complexity in the MS/MS spectra hampered identification of peaks *2*, *11*, *13*, *16* and *18*. These complexities are largely due to parent and daughter ions of co-eluting multiple constituent.

In fraction F5-4, MS spectra of the peptide eluted as peak *1* showed a molecular ion peak of 233.1499 $[M+H]^+$ which was assigned to the formula $C_{10}H_{21}N_2O_4^+$ (Δ = 1.4 ppm). After studying the diagnostic fragment ions in the MS/MS spectrum, the peptide eluted as peak *1* was identified as threonyl-leucine (TL).[36] Similarly, after comparing the MS fragment ions with reference values, the material eluted as peak *3* (221.0937 $[M+H]^+$, $C_{11}H_{13}N_2O_3^+$, Δ = 7.4 ppm)

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was tentatively assigned as 5-hydroxyltryptophan.[37] The presence of this compound was unexpected and is most likely an oxidation product of the naturally occurring tryptophan. Oxidation of amino acids and peptides during enzymatic hydrolysis is a common phenomenon.[37] The molecular ion of the peptide eluted as peak *4* was assigned to $C_{13}H_{17}N_2O_5^+$ (Δ = 1.3 ppm). This molecular formula and observed MS/MS fragment ion peaks were consistent with aspartyl-phenylalanine (DF).[38]

With retention time and MS/MS spectra similar to the peptide eluted as peak *1* of fraction F5-4, the peptide eluted as peak *5* from fraction F5-5 was also identified as TL. After studying the major fragment ions, the peptide eluted as peak *6* (203.1390 $[M+H]^+$ ($C_9H_{19}N_2O_3^+$, Δ 4.9 ppm) was identified as leucyl-alanine (LA).[39] This peptide has previously been reported to have DPP-IV inhibitory activity with an IC_{50} value of 0.091 ± 0.006 mM.[40] The fragmentation of the peptide eluted as peak *7* (461.2242 $[M+H]^+$, $C_{19}H_{33}N_4O_9^+$, Δ = 1.2 ppm) was consistent with the tripeptide leucyl-alanyl-aspartic acid (LAD) (**Figure 6A**). Similarly, after assigning characteristic *a*, *b* and *y* fragment ions, the peptide eluted as peak *8* (476.2308 $[M+H]^+$, $C_{10}H_{21}N_2O_4^+$, Δ = 1.4 ppm)) was identified as valine-glutamic acid-valine-aspartic acid (VEVD) (**Figure 6B**). The MS/MS spectra of peak *7* and *8* together with the complete fragment ion assignments are presented in **Figure 6**.

The peptides eluted as the two major peaks of fraction F5-13, peak *9* and *10*, were identified as isobaric peptides with molecular formula of $C_{12}H_{24}N_2O_3$. The fragmentation pattern of both peaks was similar and consistent with a dipeptide containing two leucine or isoleucine residues. Therefore, the two peaks were tentatively assigned as structural isomers of leucyl-leucine (LL). Both LL and IL have previously been identified from *in vitro* gastrointestinal digestion products of Brewers' spent grain protein hydrolysates as DPP-IV inhibitor.[41]

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The peptide eluted as peak *12* from fraction F5-14, was identified as a nonapeptide i.e. ETGKGEDGE, using the MaxQuant algorithm. The peptide eluted as peak *14* had similar retention time, molecular ion peak and MS fragmentation pattern as the peptide eluted as peak *10* and is therefore tentatively identified as LL. The molecular ion peak as well as fragmentation ions observed for the peptide eluted as peak *15* (279.1710 $[M+H]^+$,$C_{15}H_{23}N_2O_3^+$, $\Delta = 2.6$ ppm) were consistent with phenylalanyl-leucine (FL). This di-peptide has previously been reported as competitive inhibitor of DPP-IV with an IC_{50} value of 399.58 ± 10.81 μM.[42]

The peptide eluted as peak *17* from fraction F5-16 was identified as an octapeptide LFFSMLLML using MaxQuant. The peptide eluted as peak *19* (279.1706 $[M+H]^+$, $C_{15}H_{23}N_2O_3^+$, $\Delta = 0.9$ ppm) was observed to have the same molecular ion peak and elute at the same retention time as peak *15*. However, after a careful analysis of diagnostic fragment ions, the peptide eluted as peak *19* was identified as leucyl-phenylalanine (LF), a structural isomer of the di-peptide eluted as peak *15*. One of the characteristic differences observed between the MS/MS spectra of the peptides eluted peak *15* and *19* was the relative intensities of *m/z* 86 and 120. The base peak for LF, *m/z* 86, appear to be lower in FL, while the base peak for FL, *m/z* 120, is lower in LF. Similar diagnostic analysis of fragment ions has previously been reported as a strategy to differentiate the two structural isomers.[43]

The majority of peptides identified from the DPP-IV-inhibiting fractions were dipeptides. Several studies have suggested that dipeptides derived from dietary protein can act as potent inhibitors of DPP-IV.[26] Another interesting observation was related to terminal leucine or isoleucine residue.[44] All the five fractions with the highest DPP-IV activity was found to contain at least one peptide with leucine or isoleucine as a terminal residue. This is consistent with a previous *in silico* study which showed a general trend of high DPP-IV inhibition for peptides containing hydrophobic or aromatic amino acids at the N-terminal.[45] DPP-IV inhibitory activity

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of peptides containing amino acids, such as leucine or isoleucine at the N-terminal, is likely a result of interactions with the hydrophobic motifs of the enzyme's catalytic pockets.[46]

4. Conclusion

In the present study, peptides derived from mechanical chicken deboning residues were shown to have potential antidiabetic activity. A low molecular weight peptide fraction (F5) from SEC separation of the chicken by-product protein hydrolysate was found to inhibit DPP-IV *in vitro* and promote cellular glucose uptake *ex vivo*. A series of chromatographic fractionations and mass spectrometric analyses led to identification of the peptides constituting the DPP-IV inhibiting fractions. Common to all fractions with highest DPP-IV inhibition activity was the presence of one or more peptides with an *N*-terminal leucine or isoleucine residue. These results suggest that these peptide fractions prepared from mechanical chicken deboning residues can potentially serve as ingredients of multi-functional foods with dual effects of DPP-IV inhibition and enhancement of cellular glucose uptake.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgment

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Figure captions

Figure 1. Schematic representation of the analysis of protein hydrolysates from mechanical chicken deboning residue. Enzymatic hydrolysis experiments were performed to afford four different hydrolysates (RC80, RC240, RF80 and RF240) and after preliminary screening for DPP-IV inhibitory activity, RC80 was selected for SEC fractionation. The SEC fractionation afforded eight fractions, which were evaluated for both DPP-IV inhibitory activity and glucose uptake. Fraction F5 was further fractionated using reversed-phase chromatography, and all fractions were assessed for DPP-IV inhibitory activity. The five most potent fractions were analyzed using LC-HRMS/MS for identification of the peptides in the fractions.

Figure 2. Size exclusion chromatographic trace (at 214 nm) of RC80 (**A**) and semi-high resolution DPP-IV inhibition profile of the corresponding fractions (**B**). The DPP-IV inhibition measurements were performed in triplicate and average of the three measurements is shown

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615 with standard deviation. Plotted in red together with the chromatographic trace is retention time
616 of the molecular weight standards.

617

618 **Figure 3.** Effect of the SEC fractions (F1-F8) on cellular glucose uptake and metabolic active
619 cells. Bar plot of the relative amount of glucose uptake in cells treated with hydrolysate fractions
620 compared to control cells (i.e., cells without addition of 2DG (-2D), cells treated with stop
621 buffer before addition of 2DG (stopped), untreated cells, cells treated with 1 mM insulin) (**A**).
622 Bar plot of the amount of ATP present in cells treated with hydrolysate fractions compared to
623 untreated control cells (**B**). The data is presented as the average of at least two independent cell
624 culture experiments seeded out in triplicates ±SEM.

625

626 **Figure 4.** Reversed-phase chromatographic trace (at 214 nm) of F5 (**A**) and semi-high
627 resolution DPP-IV inhibition profile of the corresponding fractions (**B**). The DPP-IV inhibition
628 was performed in triplicate and average of the three measurements is shown with standard
629 deviation. Five fractions with DPP-IV inhibition greater than 80% were selected for further
630 analysis and are highlighted in red. Fractions with only duplicate measurements are presented
631 as an average value without standard deviation (marked with '*').

632

633 **Figure 5.** Base peak chromatograms of the five fractions F5-4 (**A**), F5-5 (**B**), F5-13 (**C**), F5-14
634 (**D**) and F5-16 (**E**).

635

636 **Figure 6.** Example of HRMS spectra obtained from LC-HRMS/MS analyses of the peptides
637 from fraction F5-13. LC-HRMS/MS spectrum of the molecular ion *m/z* 318.1667 from the

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peptide eluted as peak *7* (**A**) and the molecular ion *m/z* 461.2240 from the peptide eluted as peak *8* (**B**). After identification of characteristic fragment ions, the peptide eluted as peak *7* and *8* were identified as LAD and VEVD, respectively. Inserted to the top right are structures of the peptides with ion fragments identified in the spectrum.

Tables

Table 1. Screening of DPP-IV activity of mechanical chicken deboning residues hydrolysates.

Samples	1000 µg/mL	SD	10 µg/mL	SD
RC80	59.87	0.634	20.44	1.341
RC240	49.82	1.692	20.61	0.975
RF80	51.62	7.766	21.50	0.518
RF240	45.94	7.518	21.24	0.701

n = 3; SD: Standard Deviation

Table 2. LC-MS/MS data of identification of peptides in the DPP-IV inhibiting RP fractions. Retention time, MS and MS/MS data is presented with the sequence of the identified peptides.

Peak	Retention time (min.)	m/z $[M+H]^{n+}$ (MF, ppm)	Diagnostic fragment ions (MS/MS)	Peptides
1a	8.6	233.1499 $[M+H]^+$ ($C_{10}H_{21}N_2O_4^+$, Δ 1.4 ppm)	86.0960; 120.0670	TL
2†	9.0	476.2308 $[M+H]^+$ ($C_{10}H_{21}N_2O_4^+$, Δ 1.4 ppm)	-	-
3	9.4	221.0937 $[M+H]^+$ ($C_{11}H_{13}N_2O_3^+$, Δ 7.4 ppm)	104.0538; 133.0325; 150.0595; 173.0939	5-Hydroxytryptophan (5-HT)
4	15.4	181.1128 $[M+H]^+$ ($C_{13}H_{17}N_2O_5^+$, Δ 1.3 ppm)	120.0821	DF
5a	8.9	233.1496 $[M+H]^+$ ($C_{10}H_{21}N_2O_4^+$, Δ 5.0 ppm)	86.0968; 120.0668	TL
6	9.5	203.1390 $[M+H]^+$ ($C_9H_{19}N_2O_3^+$, Δ 4.9 ppm)	86.0968; 157.1316	LA
7	11.4	318.1667 $[M+H]^+$ ($C_{13}H_{24}N_3O_6^+$, Δ 2.2 ppm)	86.0968; 157.1346; 185.1298; 205.0834	LAD
8	18.9	461.2242 $[M+H]^+$ ($C_{19}H_{33}N_4O_9^+$, Δ 1.2 ppm)	134.0459; 233.1136; 362.1538	VEVD
9b	20.8	245.1860 $[M+H]^+$ ($C_{12}H_{25}N_2O_3^+$, Δ 3.2 ppm)	86.0974; 132.1038	LL
10b	21.2	245.1877 $[M+H]^+$ ($C_{12}H_{25}N_2O_3^+$, Δ 7.2 ppm)	86.0975; 132.1041	LL
11†	19.2	519.6880 $[M+2H]^{2+}$	-	-
12‡	20.3	461.2318 $[M+2H]^{2+}$	-	ETGKGEDGE
13†	20.6	779.7376 $[M+3H]^{3+}$	-	-
14b	21.2	245.1860 $[M+H]^+$ ($C_{12}H_{25}N_2O_3^+$, Δ 7.0 ppm)	86.0970; 132.1032	LL
15	21.7	279.1710 $[M+H]^+$($C_{15}H_{23}N_2O_3^+$, Δ 2.6 ppm)	86.0947; 120.0823; 132.1036	FL
16†	19.2	433.7220 $[M+H]^{6+}$	-	-
17‡	20.1	382.9011 $[M+H]^{3+}$	-	LFFSMLLML
18†	20.9	474.9056 $[M+3H]^{3+}$	-	-
19	21.9	279.1706 $[M+H]$ ($C_{15}H_{23}N_2O_3^+$, Δ 0.9 ppm)	86.0947; 120.0823; 166.0875	LF

All peptides identified with leucine residue (L) can interchangeably be the isobaric isoleucine (I)

[a,b] Peptides identified in more than one fraction.

†Unidentified peptides.

‡Peptides identified using MaxQuant[24].

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Figures

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Figure 1.

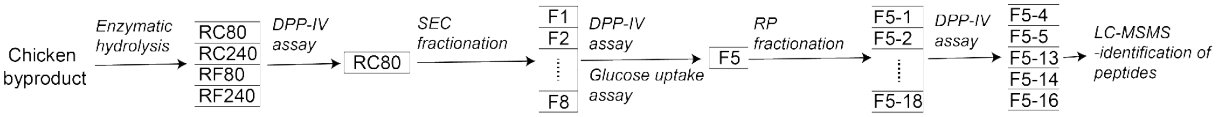
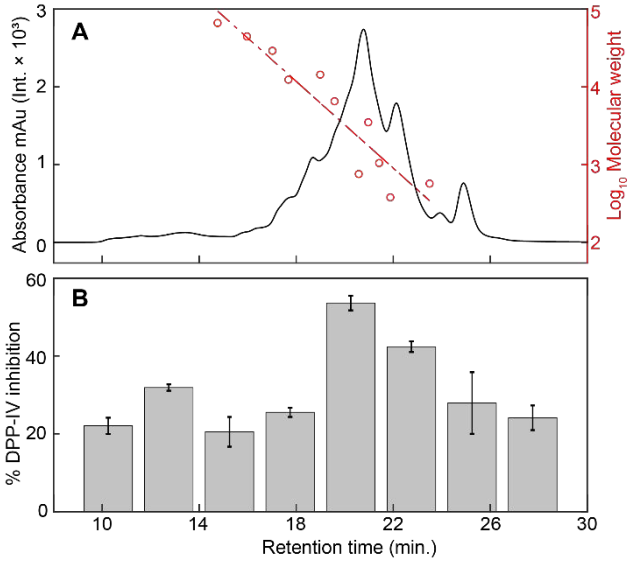


Figure 2.



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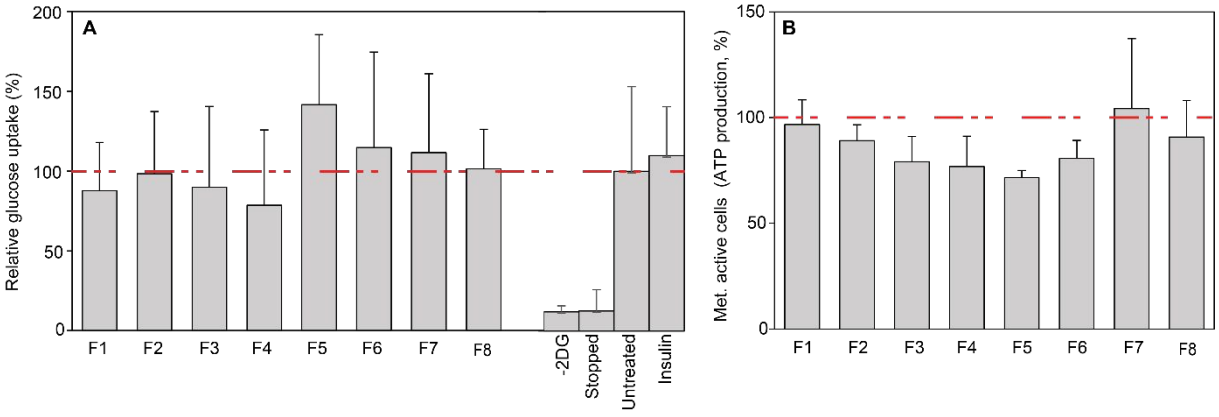

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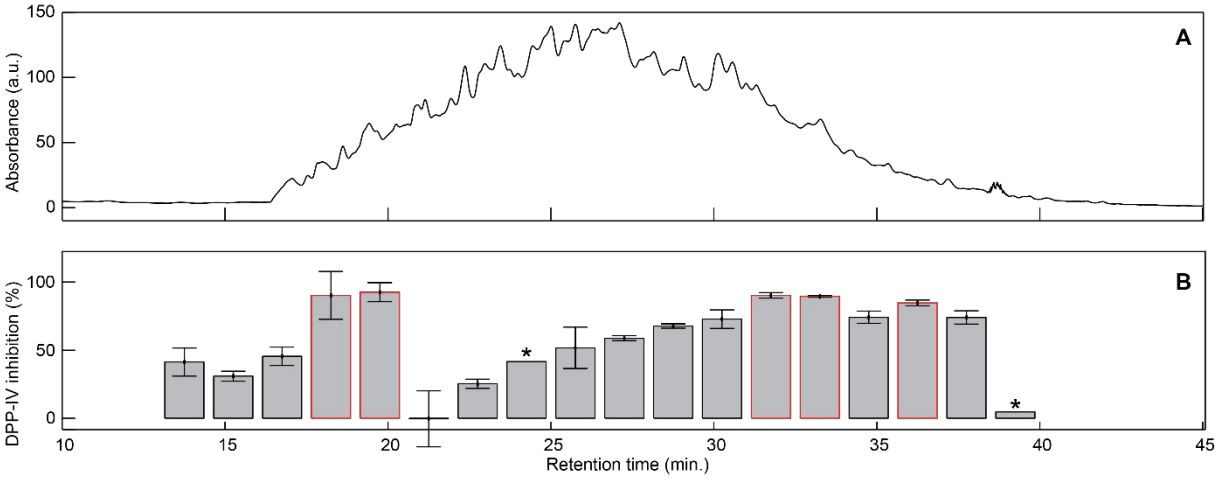
677 **Figure 4.**



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681 **Figure 5**

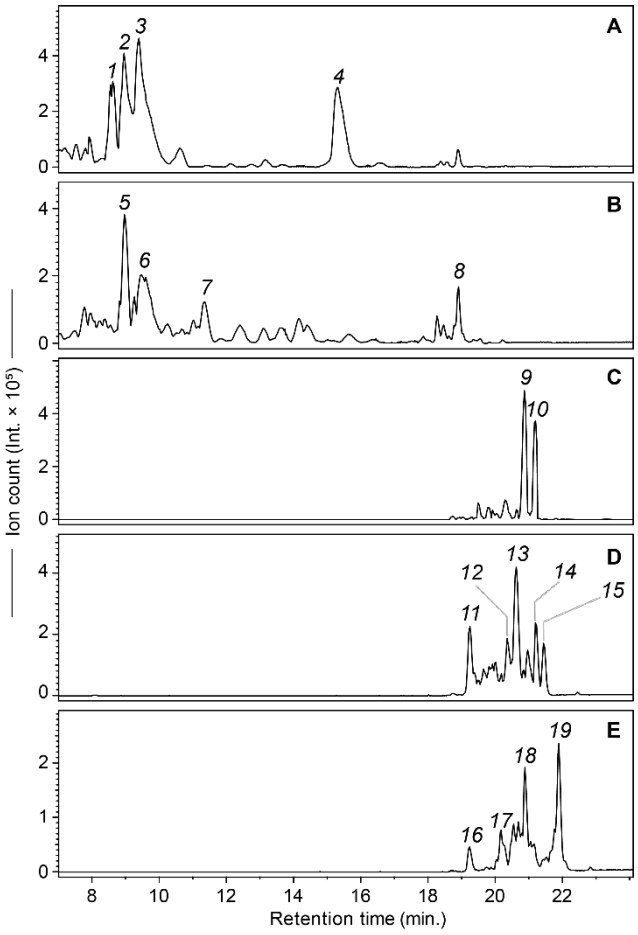

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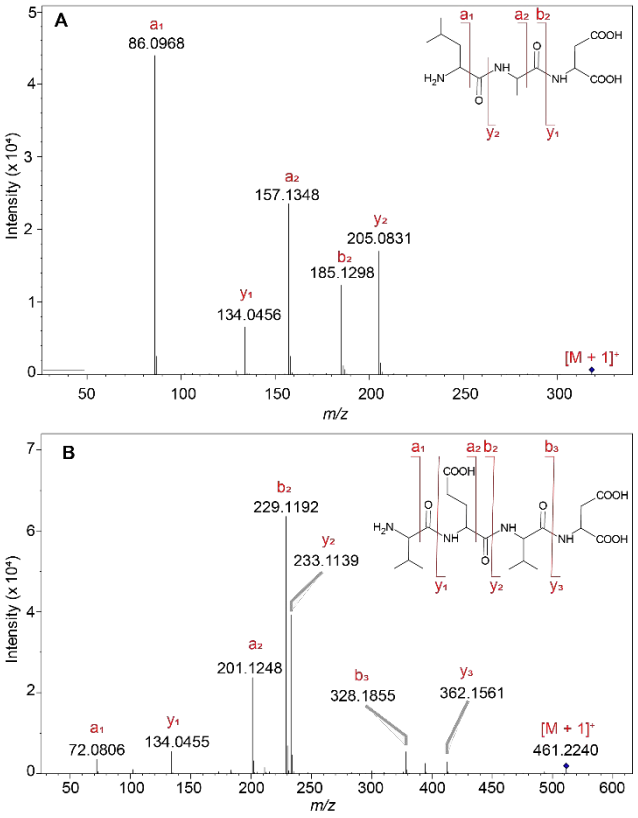
Figure 6.



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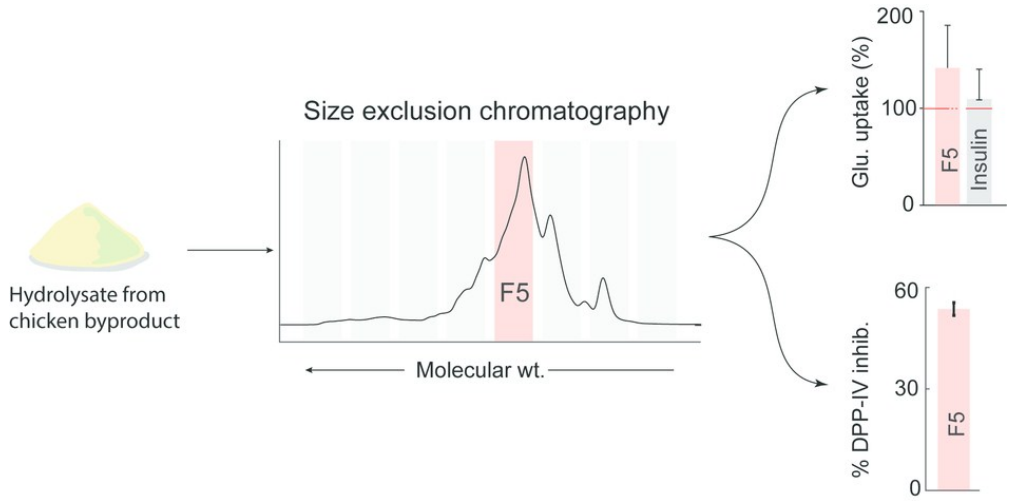

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