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# Antidiabetic effects and mechanisms of action of γ-conglutin from lupin seeds

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ABSTRACT

The glucose modulating properties of lupin have been attributed to its seed protein γ-conglutin. Here we explored the antidiabetic potential of γ-conglutin purified from lupin seeds *in-vitro*. To mimic the effects of an orally administered supplement, purified γ-conglutin was hydrolysed by gastrointestinal proteolytic enzymes and the resulting peptides evaluated for their antidiabetic effects in pancreatic β-cells and primary human skeletal muscle myotubes. γ-conglutin peptides did not promote insulin secretion in β-cells but elicited a potent insulin-mimetic action by activating insulin signalling pathways responsible for glycogen, protein synthesis, and glucose transport into myotubes. Additionally, the peptides potently suppressed the activity of DPP4 indicating their potential to increase the half-life of incretin hormones in circulation. These results substantiate the health benefits of consuming lupin seeds as part of a healthy diet and can drive the current market for lupins from primarily stockfeed, towards value-added lupin-based food products for human consumption.

## 1. Introduction

Type 2 diabetes is a combination of early insulin resistance and progressive loss of pancreatic β cell function, resulting in insufficient amounts of insulin and subsequent hyperglycaemia (Gerich, 2003). An antidiabetic agent maintains blood glucose levels either by (a) improving peripheral insulin sensitivity, (b) decreasing glucose production in the liver, (c) increasing insulin secretion from β cells or, (d) modulating the enzymatic activity of glucose metabolism-related enzymes such as dipeptidyl peptidase 4 (DPP4) and α-glucosidase (Bolen et al., 2007; Drucker & Nauck, 2006; Lebovitz, 1997). Consumption of health functional foods rich in complex carbohydrates, proteins and phytonutrients, can help in managing diabetes by promoting improved glucose homeostasis (Mirmiran, Bahadoran, & Azizi, 2014; Venkatakrishnan, Chiu, & Wang, 2019). As compared to synthetic medicines, bioactives and nutrients of plant origin are gaining attention in prevention and treatment of early stage diabetes due to their local availability, efficacy, and minimal side effects (Salehi et al., 2019; Tran, Pham, & Le, 2020).

Plants of the *Lupinus* spp, commonly known as 'lupin' (in Australia and Europe) or 'lupine' (in the Americas), are an ancient, large and diverse genus in the Fabaceae family. Out of all lupin species, only four of them are of agronomic interest: *L. angustifolius, L. albus, L. luteus*, and *L. mutabilis*. Lupins are produced for their seeds in many countries

*Abbreviations:* DPP4, dipeptidyl peptidase 4; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; RP-HPLC, reverse phase - high performance liquid chromatography; AUC, area under curve; ESI, electrospray ionisation; MS, mass spectrometry; MWCO, molecular weight cut off; DH, degree of hydrolysis; OPA, o-phthalaldehyde; PBS, phosphate buffer saline; HSMM, human skeletal muscle myotubes; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide); KRBB, Krebs ringer bicarbonate buffer; BSA, bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 2-NBDG, 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose; PI, propidium iodide; OPP, o-propargyl-puromycin; pNPG, p-nitrophenyl-β-glucopyranoside; mTOR, mammalian target of rapamycin; S6, S6 ribosomal protein; 70S6K, 70 KDa ribosomal protein S6 kinase; ERK1/2, extracellular signal-regulated kinase 1 and 2; GSK3β, glycogen synthase kinase-3β; Gαq/PLC/PKC, Gαq protein/phospholipase C/protein kinase C; IR, insulin receptor; IRS-1, insulin receptor substrate; PI3K, phosphoinositide 3-kinase (PI3K); PDK-1, 3-phosphoinositide-dependent protein kinase 1; mTORC2, mTOR complex 2; GS, glycogen synthase; TSC1/2, tuberous sclerosis complex; 4EBPI, eukaryotic initiation factor 4E binding protein 1; eIF4e, eukaryotic initiation factor 4E; GLP-1, glucagon like peptide 1; GIP, gastric inhibitory peptide.

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including Australia, Denmark, Chile, Poland, Germany, USSR, USA, and Ukraine (Abraham et al., 2019). The majority of lupin seed production is consumed as ruminant, swine and poultry feed (OGTR, 2013). Lupin has high protein and fibre content with negligible starch, and thus can lower the glycaemic load of the diet (AgriFutures, 2017; Duranti, 2006). Clinical studies in animals and humans indicating hypoglycaemic actions of lupin seed-based food preparations and/or proteins have been reported.(Capraro et al., 2014; Dove et al., 2011; Hall, Thomas, & Johnson, 2005; Keogh, Atkinson, Eisenhauer, Inamdar, & Brand-Miller, 2011; Lee et al., 2006, 2006). Commercial interest in lupin seed as a health functional food ingredient and nutraceuticals has increased due to its low cost, non-genetically modified status, and low levels of potentially hazardous phytoestrogenic compounds (DAFWA, 2018). In addition, modern genotypes of *L. angustifolius* produced in Australia are known as Australian sweet lupin due to the vastly reduced levels of alkaloids in the seeds compared to the original forms.

The glucose modulating property of lupin seed has been attributed to a specific seed protein component, γ-conglutin, as reported in multiple cellular and animal models, as well as one human post-prandial trial (Bertoglio et al., 2011; Capraro et al., 2013; González-Santiago et al., 2017; Magni et al., 2004; Muñoz, Luna-Vital, Fornasini, Baldeón, & Gonzalez de Mejia, 2018; Rosa Lovati et al., 2012; Terruzzi et al., 2011; Vargas-Guerrero, García-López, Martínez-Ayala, Domínguez-Rosales, & Gurrola-Díaz, 2014). Different studies have attributed the hypoglycaemic action of γ-conglutin to its insulin-mimetic properties (Muñoz et al., 2018; Rosa Lovati et al., 2012; Terruzzi et al., 2011). Gamma-conglutin was shown to activate intracellular kinases and adaptor proteins involved in insulin signalling, promote translocation of GLUT-4 receptors to the cell membrane, and regulate muscle-specific gene transcription similar to insulin *in vitro*. (Terruzzi et al., 2011) However, the end effects of augmented protein synthesis and glucose uptake were not directly assessed in previous studies. Also, the proposed mechanism of action has only been investigated in mouse immortalised C2C12 myoblasts without further validation in primary human cells that would represent a much more physiological and translatable model. Recently, we have demonstrated that the hydrolysate of lupin protein extract can directly stimulate insulin secretion from pancreatic β cells, therefore operating as an insulinotropic agent via a $G\alpha_q$ protein signal transduction ($G\alpha_q$ protein/phospholipase C/protein kinase C - $G\alpha_q$/PLC/PKC pathway) (Tapadia, Carlessi, Johnson, Utikar, & Newsholme, 2019). However, we did not establish, if γ-conglutin in the extract was the protein responsible for the insulin secretory action. Furthermore, bioactive peptides obtained from dietary proteins have been reported to modulate insulin secretion and glucose metabolism through inhibition of DPP4 and α-glucosidase enzymes. Inhibitors of DPP4 and α-glucosidase enzyme are currently being used in the management of type 2 diabetes (Abbas, Al Harrasi, Hussain, Hamaed, & Supuran, 2019). The enzyme DPP4 increases the circulating levels of incretins in the blood, which further stimulates secretion of insulin from pancreatic β cells (Ahrén, 2007). The enzyme α-glucosidase in the human gastrointestinal tract hydrolyses starch and disaccharides into glucose monomers (Ibrahim, Bester, Neitz, & Gaspar, 2018). Thus, inhibitors of these enzymes directly or indirectly alleviate the rise in blood glucose levels. It was observed that soy and lupin derived peptides inhibited the activity of DPP4 in an *in-vitro* screening bioassay (Lammi, Zanoni, Arnoldi, & Vistoli, 2016). These peptides also inhibited the enzyme in human intestinal Caco-2 cells and *ex-vivo* in human serum (Lammi et al., 2018). The effects of lupin/γ-conglutin protein or peptide(s) on the activity of α-glucosidase enzyme have not yet been addressed.

Accurate identification, quantification, and characterization of proteins purified from natural sources is an essential step in determining their biological activity. Yet, in most of the studies on the biological activity of γ-conglutin, the purity of the γ-conglutin enriched preparation has either not been reported, or has only been determined by semi-quantitative SDS-PAGE analysis (Bertoglio et al., 2011; Capraro et al., 2013; González-Santiago et al., 2017; Magni et al., 2004; Muñoz et al., 2018; Rosa Lovati et al., 2012; Terruzzi et al., 2011; Vargas-Guerrero et al., 2014). The γ-conglutin purity in these preparations i.e. γ-conglutin enriched lupin protein isolate (type F fraction), γ-conglutin enriched dry extract (Pro-Gamma™) and laboratory scale purified γ-conglutin was reported as 35%, 60% and 90% of total proteins respectively based on SDS-PAGE densitometric scanning (Bertoglio et al., 2011; Duranti, Sessa, Scarafoni, Bellini, & Dallocchio, 2000; Magni et al., 2004; Rosa Lovati et al., 2012). Moreover, the actual purity of γ-conglutin based on dry solid basis might be lower than the mentioned purity. Thus, there is a need for achieving high purity γ-conglutin that is well-characterized using appropriate quantitative analytical methods.

Despite some recent advances, the molecular mechanism of action of γ-conglutin as an insulinotropic and/or insulin-mimetic agent, and as a potential enzyme inhibitor, still needs to be systematically investigated and validated in meaningful physiological models. A process for purification of γ-conglutin from lupin seed extract was previously developed in our laboratories (Mane, Bringans, Johnson, Pareek, & Utikar, 2017). In the present study, the previously developed process was used for production of γ-conglutin that on characterization of the purified protein fraction gave ≥ 95% γ-conglutin purity (w/w of total proteins in dry solid). To evaluate potential efficacy of this γ-conglutin preparation as an oral intake supplement with hypoglycaemic action, hydrolysis was carried out in an *in vitro* gastrointestinal proteolytic digestion model. Then, the effects of the resulting peptides were evaluated for (a) insulinotropic (insulin secretion) action in pancreatic β cells (BRIN-BD11 and INS-1E) (b) insulin-mimetic action (insulin signalling, glucose uptake and protein synthesis) in primary human skeletal muscle myotubes, and (c) inhibition of DPP4 and α-glucosidase enzymatic activities.

## 2. Materials and methods

### 2.1. Materials

Mature seeds of *L. angustifolius* (cv. Coromup) were obtained from the Department of Primary Industries and Regional Development (South Perth, Western Australia). All chemicals were procured from Sigma Aldrich (NSW, Australia), unless indicated otherwise. TGX precast polyacrylamide gels, Laemmli sample buffer (4X), Broad Range Marker protein standards and Clarity™ Western enhanced chemiluminescence (ECL) substrate were purchased from BioRad Laboratories Inc. (California, USA). Ultrasensitive rat insulin enzyme-linked immunosorbent assay (ELISA) kit from Mercodia (Uppsala, Sweden). Click-iT® Plus OPP Alexa Fluor® 594 Protein Synthesis Assay Kit, Amplex® red glucose/glucose oxidase assay kit, Quant-iT™ PicoGreen™ ds DNA assay and Pierce™ BCA Protein Assay Kit were acquired from Thermofisher Scientific (Massachusetts, USA). DPP4 Inhibitor Screening Assay Kit was obtained from Cayman Chemical (Michigan, USA). Polyclonal goat anti-rabbit immunoglobulins/HRP from Dako (Glostrup, Denmark) and protease/phosphatase inhibitor cocktail (100X) and primary rabbit mAb (p-mTOR (Ser 2448; #2971S), p-AKT (Ser 473, #9271S), p-S6 (Ser235/236, #4858S), p-GSK3B (Ser9; 9336S), p-ERK1/2 (#4370), GAPDH (#D16H11), mTOR (#2972S), AKT (#9272S), S6 (#2217S), GSK3B (#9315S), ERK1/2 (#4695)) were obtained from Cell Signaling Technology (Massachusetts, USA). RPMI-1640, DMEM F12, Fetal Bovine Serum (FBS) from sourced from HiMedia Laboratories (Mumbai, India). Skeletal muscle cell growth medium kit was obtained from Lonza (Basel, Switzerland).

### 2.2. Extraction and purification of γ-conglutin

Lupin proteins were extracted from defatted *L. angustifolius* kernel seed flour as described by S. Mane, Agrawal, and Utikar (2014). The γ-conglutin was purified from lupin extract by using combination of chromatographic steps previously developed in our laboratory (Mane et al., 2017). The γ-conglutin fractions were obtained from three

replications of the purification processes. These triplicate samples were analysed individually for characterization purposes. Then the purified γ-conglutin triplicate samples were pooled into one, desalted using HiPrep 26/10 desalting column (Cytiva, Danaher Corporation Life sciences, Washington DC, USA), sterile filtered and aliquots were stored at −20 °C.

### 2.3. Protein profile by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The purified γ-conglutin was diluted with 4X Laemmili sample buffer. To prepare reduced samples, β-mercaptoethanol was added to the sample and heated at 99 °C for 10 min. The SDS-PAGE analysis was performed using pre-cast gels (BioRad Laboratories Inc., Hercules, USA), for optimal resolution for proteins between 10 and 100 KDa, in a Mini-Protean tetra cell electrophoresis assembly (BioRad Laboratories Inc., Hercules, USA) at 100 V. After staining the gels, they were scanned and analysed using the Molecular Imager® Gel Doc™ XR System v5.2.1 (BioRad Laboratories Inc., California, USA) tool (Heukeshoven & Dernick, 1985). The molecular weight of each band in sample was calculated by comparing band migration with the molecular weight of marker proteins (Broad range SDS-PAGE molecular weight marker (BioRad Laboratories Inc., California, USA that was ran in each gel). Intensity (% of total band intensity) of each band was analysed by densitometric scanning using Image Lab 4 software (BioRad Laboratories Inc., California, USA).

### 2.4. Immunodetection of γ-conglutin using Western blot

Proteins in lupin total protein extract and purified γ-conglutin fractions were separated by SDS-PAGE and electrotransferred to PVDF membranes by the wet transfer method in a Mini Trans-Blot cell tank (Bio-Rad Laboratories Inc., California, USA) (Yang & Mahmood, 2012). Then, membranes were blocked with 3% skim milk for 60 min and then incubated overnight at 4 °C with a γ-conglutin primary antibody (1:1000 dilution) raised in rabbit against a synthetic peptide of γ-conglutin (heavy subunit – 30 KDa). This antibody was a kind gift from Prof. Karam Singh (Director of Centre for Crop and Disease Management, Curtin University, Western Australia). Membranes were washed and then incubated in horseradish peroxidase (HRP) conjugated secondary antibody (Agilent Dako, California, USA) for 60 min. Clarity™ Western ECL substrate was used to detect target proteins on the blots. The molecular weight of γ-conglutin bands were calculated by comparing band migration with molecular weight of marker proteins (Precision Plus Protein™ Kaleidoscope™ Prestained protein standards, BioRad Laboratories Inc., California, USA) that was ran in each gel. Immunoreactive protein bands were visualized and analysed (% band intensity) using Molecular Imager® Gel Doc™ XR System v5.2.1 (Bio-Rad Laboratories Inc., California, USA).

### 2.5. Determination of γ-conglutin purity by reverse–phase high performance liquid chromatography (RP-HPLC)

A RP-HPLC method for detection and quantification of γ-conglutin (Mane et al., 2017) was modified (Mane et al., 2017). Agilent Technologies (California, USA) 1200 series HPLC system with diode array detector was used for analysis with a Zorbax 300SB C-18 column (4.6 × 250 mm), 300 Å pore size (Agilent Technologies, USA). The column was equilibrated with trifluoroacetic acid (0.1%) in water at pH 2.2. Elution of proteins was carried out by a continuous gradient of 0–100% elution buffer (0.1% trifluoroacetic acid in acetonitrile) for 20 min. The injection volume and flowrate were 50 μl and 0.8 ml/min respectively throughout the run. A wavelength of 280 nm was used to detect proteins and peak integration (for calculating area under curve - AUC) was performed by Chemstation software (Agilent Technologies, California, USA). The samples were analysed in triplicates and percent purity of γ-conglutin was calculated as the percent of the γ-conglutin AUC of the total chromatographic peaks AUC. The purity of γ-conglutin was reported as w/w of total proteins at 280 nm.

### 2.6. Identification of γ-conglutin by mass spectrometry (MS)

The purified desalted γ-conglutin fraction was digested with trypsin and extracted according to a standard sample preparation method in LC-MS/MS analysis. (Bringans et al., 2008) Briefly, trypsin (12.5ug/ml) digest solution (10ul) was added to the samples followed by overnight incubation at 37 °C. The digested peptides were extracted with acetonitrile containing 1% TFA (20 min incubation). Peptides were analysed by electrospray ionisation (ESI) mass spectrometry using the Shimadzu Prominence nano-HPLC system coupled with 5600 Triple TOF mass spectrometer (Sciex, Victoria, Australia). The tryptic peptides were loaded on to Zorbax 300SB-C18 column (Agilent Technologies, USA). The peptides were separated with a linear gradient of water (0.1% formic acid) and acetonitrile (0.1% formic acid). Temperature and voltage applied between needle and source in the ESI mass spectrometer were 350 °C and 2050 V respectively. For MS/MS analysis, scan rate and spectra collection rate were 1 spectra/s and 1000 ms/spectra respectively. The data were acquired in an information-dependent acquisition (IDA) mode with Analyst TF 1.6 software (AB Sciex). The MS settings were as follows: Ionspray Voltage Floating (ISVF) = 2300 V, curtain gas (CUR) = 20, ion source gas 1 (GS1) = 20, interface heater temperature (IHT) = 150, and declustering potential (DP) = 70 V. The first TOF MS scan was performed in the mass range of 400–1250 Da with a 0.25 s TOF MS accumulation time, whereas the MS/MS product ion scan was performed in the mass range of 100–1800 Da with a 0.1 s accumulation time. The criteria for product ion fragmentation was set as follows: ions ($>$400 $m/z$ and $<$ 1250 $m/z$) with charge states of 2 to 5 and an abundance threshold of $>$ 250 cps. Former target ions were excluded for 10 s after one occurrence. The maximum number of candidate ions per cycle was 20 spectra. IDA advanced "rolling collision energy (CE)" was applied for subsequent MS and MS/MS scans. Spectra were analysed for protein identification using Mascot sequence matching software (Matrix Science, Boston, USA) in MSPnr100 database with Viridiplantae taxonomy. The samples were analysed in duplicates.

### 2.7. Preparation of γ-conglutin hydrolysate

Purified γ-conglutin protein was hydrolysed using enzyme pepsin and pancreatin (United States Pharmacopoeia reference standard, Sigma, Merck, Dermstadt, Germany) in two stages to mimic gastrointestinal digestion conditions. The γ-conglutin protein was hydrolysed with pepsin (pH 2) for 3 h followed by hydrolysis with pancreatin (pH 7.5) for another 4 h (Tapadia et al., 2019). After the hydrolysis process, the enzymes were removed from hydrolysate by ultrafiltration using a 3 KDa molecular weight cut-off (MWCO) Minimate™ tangential flow filtration membrane cassette (Pall Corporation, Port Washington, USA). The permeate containing the peptide fragments was sterile filtered, aliquoted and stored at −20 °C. A complete peptide profile of hydrolysate was obtained from mass spectrometry analysis as detailed in Section 2.6, except, the hydrolysate was not further digested with trypsin enzyme in LC-MS/MS sample preparation step.

### 2.8. Degree of hydrolysis (DH)

Samples were withdrawn every 30 min during the hydrolysis process and the protein DH was determined using the o-phthalaldehyde (OPA) method (Spellman, McEvoy, O'Cuinn, & FitzGerald, 2003). The OPA reagent consisted of disodium tetraborate decahydrate (0.1 M), sodium dodecyl sulphate (1 mg/ml), OPA (0.80 mg/ml) and dithiothreitol (0.88 mg/ml). In this assay, 50 μl of the sample was diluted with 250 μl phosphate buffer saline (PBS) and mixed with 3 ml OPA reagent. Primary amines, generated due to hydrolysis, react with OPA to give a

complex absorbing in the UV range. After 5 min of incubation, absorbance at 340 nm was measured in UV–visible spectrophotometer (Jasco V-670, Tokyo, Japan). The amino nitrogen content was calculated from the standard curve equation of 0–2 mM L-serine (absorbance at 340 nm Vs L-serine concentration). DH was calculated according to the following equation: (Adler-Nissen, 1979)

$$DH(\%) = \frac{(N_2 - N_1) \times 100}{N_{pb}}$$

where $N_1$ is amino nitrogen content of γ-conglutin, $N_2$ is amino nitrogen content of peptides in hydrolysate at particular sample withdrawal time, and $N_{pb}$ is nitrogen content in peptide bonds.

### 2.9. Cell culture

The INS-1E cells were a kind gift from Prof. Marco Falasca (Curtin Health Innovation Research Institute, Curtin University, Australia). The INS-1E cells were maintained in RPMI-1640 medium supplemented with 15% FBS, 1% penicillin–streptomycin, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 10 mM HEPES, pH 7.4. BRIN-BD11 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 1% penicillin–streptomycin, pH 7.4. Both the cell lines were cultured at 37 °C in a humidified atmosphere of 5% $CO_2$. The cells were sub-cultured in 3–4 days and the media was changed once after 2 days. BRIN-BD11 and INS-1E used in the experiments were between 20 and 32 and 11–15 passages respectively. Primary human skeletal muscle myoblasts (Lonza, Switzerland; Cat. No. CC-2580) had been isolated from the upper arm or leg muscle tissue of a healthy female donor. The myoblasts were propagated in skeletal muscle growth medium-2 supplemented with human epidermal growth factor, FBS, L-glutamine, gentamicin/amphotericin-B, dexamethasone. The media was changed after 48 h until 60–70% cell confluency was achieved. Supplier's (Lonza) instruction for the differentiation process of myoblasts into myotubes was followed by replacing the growth media with differentiation media DMEM F-12 medium containing horse serum (2%), penicillin–streptomycin (1%), pH 7.4 at 37 °C in a humidified atmosphere of 5% $CO_2$. The differentiation media was changed after every 48 h and the cells were cultured for 6 days until multinucleated myotubes were observed. Primary human skeletal muscle myotubes (HSMM) used in all experiments were between 4 and 8 passages.

### 2.10. Cell viability

Approximately 10,000 cells/well were seeded in a 96 well plates and incubated at 37 °C overnight. Next day, the cells were incubated with γ-conglutin peptides (20–500 μg/ml) in media. After 24 h of incubation, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (5 mg/ml; 20 μl per 200 μl medium) was added to all the wells and the cells were incubated with MTT for further 4 h. Subsequently, the media was removed and dimethylsulfoxide was added to solubilise formazan crystals. The plates were shaken thoroughly, and absorbance was read at 570 nm in AN Ensight Multimode plate reader (Perkin Elmer, Massachusetts, USA). The analysis was performed in triplicates. Cell viability of treated cells (% of control) was calculated by comparing absorbance of treated cells with absorbance of control cells (100% viable cells).

### 2.11. Acute insulin secretion assay

The BRIN-BD11 and INS-1E cells were seeded in 96 well plates (20,000 cells/well) and allowed to attach overnight. Next day, cells were washed and pre-incubated with Krebs Ringer bicarbonate buffer (KRBB) supplemented with 1.1 mM glucose for 40 min at 37 °C. Then, cells were incubated with γ-conglutin peptides (20–200 μg/ml) in KRBB (16.7 mM glucose) for 20 min at 37 °C followed by centrifugation. Supernatant aliquots were removed and stored at −20 °C. Insulin content in the supernatant (insulin secretion μg/L) was quantified using ultrasensitive rat insulin ELISA kit (Mercodia, Sweden). The primary outcomes of insulin secretion obtained from each well were normalised with the total cell protein content of that respective well. The results from three or more independent experiments were pooled and reported as fold change in insulin secretion compared to control (16.7 mM glucose).

### 2.12. Insulin signalling

Differentiated HSMM in T75 flasks were serum starved overnight. Next day, the cells were treated either with insulin (100 nM) or different concentrations of γ-conglutin peptides (2–200 μg/ml) for 20 min. The cells were then lysed in radioimmunoprecipitation buffer (RIPA) containing 1% protease and phosphatase inhibitors cocktail followed by sonication and total protein content was quantified using bicinchoninic acid (BCA) assay method. (Brown, Jarvis, & Hyland, 1989) Cell protein extracts were separated by SDS-PAGE and electrotransferred on nitrocellulose membrane. The membranes were blocked with 3% bovine serum albumin (BSA) for 60 min and incubated overnight at 4 °C with the following rabbit phosphorylated and total primary antibodies (1:1000 dilution): protein kinase B/Akt (Ser473), mammalian target of rapamycin (mTOR) (Ser2448), S6 ribosomal protein (Ser235/236) protein, 70 KDa ribosomal protein S6 kinase (70S6K) (Thr389), extracellular signal-regulated kinase 1 and 2 (ERK1/2) (Thr202/Tyr204), glycogen synthase kinase-3β (GSK3β) (Ser9). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping protein. Further immunodetection analysis steps were performed as previously detailed in Section 2.4. The analysis was performed in triplicates.

### 2.13. Cellular glucose uptake

Myoblasts (100,000 cells/well) were seeded in 6 well plates. The cells were allowed to grow and differentiate for 6 days. On the day 7, myotubes in each well were washed and challenged for 30 min with glucose free - DMEM media containing 20 μM of 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose (2-NBDG) (fluorescent deoxy glucose analog) with and without γ-conglutin peptides (20–200 μg/ml) or insulin (100 nM). Negative control wells with γ-conglutin peptides/insulin were treated in a similar manner but without 2-NBDG. Plates were incubated at 37 °C. Later, the cells were trypsinised, centrifuged and resuspended in cold PBS containing 1 μg/ml propidium iodide (PI) used for detecting dead cells. For each sample, 10,000 live cells events were recorded in FACS LSR Fortessa flow cytometer (BD Biosciences, Heidelberg, Germany). After gating (allotting graphical boundary/area) single and PI negative cells, forward scatter, side scatter and 2-NBDG median fluorescence intensities were analysed using FlowLogic FCS analysis software (Inivai Technologies, Melbourne, Australia). The fluorescence intensities (2-NBDG uptake by cells) of treated cells were normalised with the intensity obtained from control cells and fold increase in mean values of treatments with respect to control were reported.

### 2.14. Glycogen content

The differentiated HSMM cells, in T75 flasks, were serum starved overnight. Next day, the cells were incubated in glucose-free DMEM media for 2 h (glucose starvation step). Later, the cells were treated with insulin and γ-conglutin peptides (200 μg/ml and 20 μg/ml) in DMEM high glucose media for 2 h. The cells were trypsinised, centrifuged, and resuspended in 1 ml cold PBS containing 0.5% BSA and transferred to microcentrifuge tubes. An aliquot (50 μl) of the cell suspension from each tube was removed and lysed with RIPA for DNA quantification using Quant-iT™ PicoGreen™ ds DNA assay (Thermofisher Scientifics, Massachusetts, USA). The remaining cell suspensions were resuspended in 2 M HCl (100 μl). The micro-centrifuge tubes containing the samples

were sealed and heated at 99 °C for an hour. Then the acidic suspension in micro-centrifuge tubes was neutralised with 2 M NaOH. The cell suspension was centrifuged and glucose content in the supernatant of each sample tube was determined using Amplex® red glucose/glucose oxidase assay kit (Thermofisher Scientifics, Massachusetts, USA). Glucose content in each cell lysate was normalised with its respective DNA content. The glucose content corresponded to the glucose stored within cells in the form of glycogen units. The glucose content in the treated cell lysates was normalised with glucose content of control and fold change in glycogen measurement with respect to control was reported.

### 2.15. Protein synthesis assay

The manufacturer's protocol for Click-iT® Plus OPP Alexa Fluor® 594 Protein Synthesis Assay Kit (Thermofisher Scientifics, Massachusetts, USA) was followed to analyse synthesis of nascent cellular proteins. In brief, myoblasts were seeded (150,000 cells/well) in 6 well plates containing glass coverslips coated with 0.5 mg/ml poly-D-lysine. The cells were differentiated into myotubes in these wells. After overnight serum starvation, cells were incubated in DMEM media containing Click-iT® O-propargyl-puromycin (OPP) reagent (10 μM) in the presence and absence (control) of treatments: insulin (100 nM), γ-conglutin peptides (20–200 μg/ml) and cycloheximide (10 μM) (negative control) for 30 min. After incubation, the cells were washed and fixed with paraformaldehyde (4%) for 15 min. The cells were permeabilised with Triton®X-100 (0.5%) for 15 min. Next, the cells were incubated in the Click-iT® OPP reaction cocktail (reagents - copper protectant, Alexa Fluor® 594 picolyl azide, reaction buffer additive) for 30 min in dark followed by staining with HCS NuclearMask® Blue to measure DNA content. The OPP conjugated nascent polypeptide chains were fluorescently labelled with AF594 - AlexaFluor®594 picolyl azide for visualisation by fluorescence microscopy. The cells were washed with PBS and mounted in Prolong® Antifade Diamond mountant (Thermofisher Scientifics, Massachusetts, USA) protected from light. The next day, each mounted sample was imaged by confocal microscopy on Nikon A1+ microscope with 20X objective (Nikon, Tokyo, Japan). Images were captured using NIS Elements image acquisition software (Nikon, Tokyo, Japan) and analysed using ImageJ software (Schneider, Rasband, & Eliceiri, 2012). The same global threshold values were set for analysing all the AF594 fluorescent red images (nascent protein synthesis). 'Pixel integrated density per unit area (mean)' within the set threshold window was obtained. In each experiment, at least 15 images per sample were obtained. Three independent experiments were performed. The image mean value of cells unlabelled with OPP (blank) was subtracted from the image mean value of treated/control cell samples labelled with OPP. The fold increase in mean values of treatments with respect to control (unstimulated serum starved cells) were reported.

### 2.16. DPP4 inhibition assay

The DPP4 inhibition assay was carried out in triplicate in half volume 96-well solid white plates. As per the DPP4 inhibitor screening assay kit manufacturer's protocol, the reaction mixture was prepared by mixing tris-HCl (20 mM) assay buffer containing NaCl (100 mM) and EDTA (1 mM), DPP4, sample (γ-conglutin protein/peptides) or positive control - sitagliptan (100 μM). The reaction was subsequently started by adding substrate gly-pro-aminomethylcoumarin (5 mM) and incubated at 37 °C for 30 min. Fluorescence signals were measured in a multimode plate reader (Ensight, Perkin Elmer) using excitation and emission wavelength of 350 nm and 460 nm respectively. Control (100% initial activity) was measured by the addition of vehicle instead of inhibitors (sample or positive control). Background absorbance of samples without enzyme and substrate were also measured. DPP4 activity of (%) sample/inhibitor was calculated as:

$$DPP4\ activity\ (\%) = \frac{(sample\ or\ inhibitor\ fluorescence - background\ fluorescence) \times 100}{(Control\ fluorescence - background\ fluorescence)}$$

### 2.17. α-glucosidase inhibition assay

α-glucosidase inhibition assay was performed according to standard methods with minor modifications (Elya et al., 2012). In brief, the reaction mixture was prepared by adding following reagents: sodium phosphate (50 mM) pH 6.86 buffer, α-glucosidase enzyme (0.15 U/ml) containing BSA (0.2%), sample (γ-conglutin protein/peptides) or positive control - acarbose (50 mM). The reaction was subsequently started by adding substrate p-nitrophenyl-β-glucopyranoside (pNPG) (5 mM) and incubated at 37 °C for 15 min. Later, sodium carbonate (200 mM) was added to stop the reaction. α-glucosidase activity was determined by measuring the absorbance at 400 nm in multimode plate reader (Ensight, Perkin Elmer). Background absorbance of samples without enzyme and substrate pNPG were also measured. α-glucosidase activity (%) of sample/inhibitor was calculated as:

$$\alpha\ glucosidase\ activity\ (\%) = \frac{(sample\ or\ inhibitor\ absorbance - background\ absorbance) \times 100}{(Control\ absorbance - background\ absorbance)}$$

### 2.18. Statistical analysis

GraphPad Prism v.6.0 software was used to perform statistical analysis. The results were reported as mean ± standard deviation (SD) of at least three independent experiments. The datasets were analysed by ANOVA (comparison between three or more groups) with a Dunnett post-hoc test or independent sample *t*-test (comparison between two groups). Statistical significance was set at $p \leq 0.05$.

## 3. Results

### 3.1. Biochemical characterisation of purified γ-conglutin

Purified chromatography elution fractions containing γ-conglutin

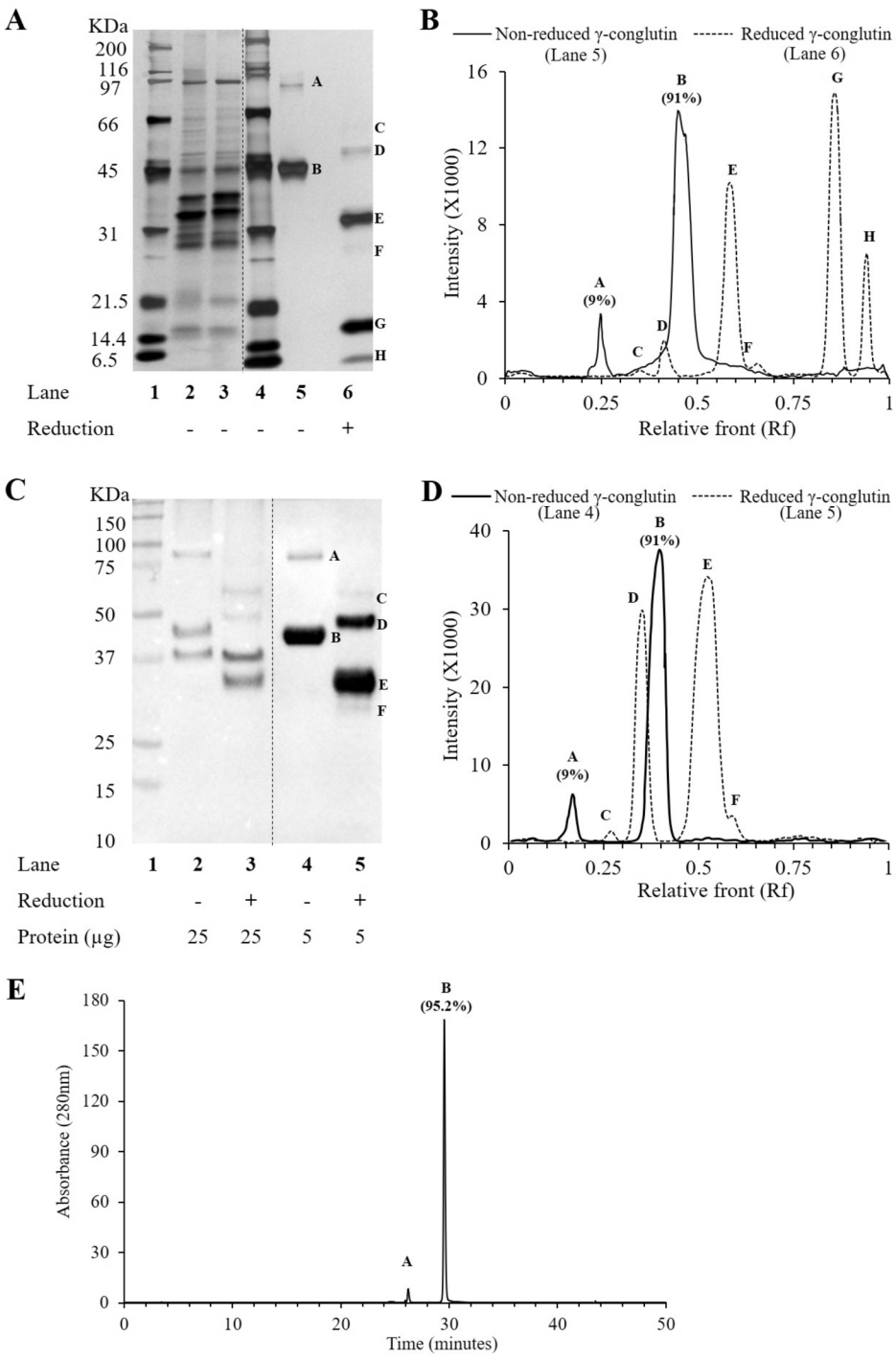


**Fig. 1. Biochemical characterization of purified lupin protein, γ-conglutin:** (A) SDS-PAGE profile of molecular weight marker (lane 1 and 4), extract at pH 4 (lane 2), chromatographic column feed (lane 3), purified γ-conglutin, non-reduced (lane 5) and purified γ-conglutin, reduced (lane 6); (B) SDS-PAGE densitogram of non-reduced and reduced purified γ-conglutin; (C) Western blot of non-reduced (−) and reduced (+) extract (lane 2 and 3) and pure γ-conglutin (lane 4 and 5); Amount of protein loaded in lane 2–3 is 25 μg and lane 4–5 is 5 μg (D) Western blot densitogram of non-reduced and reduced purified γ-conglutin; (E) RP-HPLC analysis of purified γ-conglutin fraction obtained from the chromatographic purification process.

were pooled and desalted for identification and characterization using SDS-PAGE, Western blot, RP-HPLC, and MS/MS. SDS-PAGE analysis of extract, column feed, and pure γ-conglutin are described in Fig. 1A. Along with γ-conglutin, proteins with different molecular weights (10–100 KDa) were present in the extract (lane 2) and chromatographic feed (lane 3). Lane 5 represents the purified γ-conglutin fraction obtained in the elution step of chromatography. The transition from tetramer (200 KDa) to dimer (90–100 KDa) to monomer (45–48 KDa) occurs when pH shifts from neutral to acidic (pH 4.5) (Capraro, Spotti, Magni, Scarafoni, & Duranti, 2010). Due to pH modifications in the extraction and purification processes, γ-conglutin existed in two forms: dimer of 91KDa (Lane 5, band A) and monomer of 45–48 KDa (Lane 5, band B). Each γ-conglutin monomer is composed of a heavy subunit (30 KDa) and a light subunit (17 KDa) linked together by disulphide bonds (Restani, Duranti, Cerletti, & Simonetti, 1981). The presence of γ-conglutin monomer and dimer was confirmed by analysing the reduced γ-conglutin gel profile (Lane 6; bands E and G). Under reducing conditions, disulphide bonds in monomeric and dimeric γ-conglutin were cleaved to give its heavy and light polypeptides subunits. Moreover, during sample preparation (for reduced samples), the high temperature (99 °C for 10 min) induced aggregation of uncleaved γ-conglutin or incomplete degradation of γ-conglutin dimers and monomers (bands C, D, F and H). Densitometric analysis of reduced and non-reduced purified γ-conglutin fraction was performed to measure the intensity of the

protein bands (Fig. 1B). In lane 5, except for the two bands at 91 KDa (9% band intensity) and 45 KDa (91% band intensity), no other protein bands were visible indicating ~100% γ-conglutin purity.

To further confirm the identity of γ-conglutin, Western blot analysis was performed. The anti γ-conglutin antibody has specificity to the heavy subunit of γ-conglutin (~30 KDa) (Fig. 1C). Monomeric (45 KDa) and dimeric (91 KDa) bands of γ-conglutin were visible in the lupin protein extract (before purification) (lane 2). This extract, under reducing condition (lane 3), displayed a heavy subunit band of γ-conglutin (30 KDa) and two aggregate bands (~48–50 KDa and ~60 KDa). High temperature stress condition during preparation of reduced sample might have induced protein aggregation or change in conformation resulting into high protein molecular weight bands. Apart from the γ-conglutin in the extract, the antibody also showed non-specific binding to a protein at 37 KDa in both lanes (2 and 3). Pure γ-conglutin fraction (lane 4) showed the presence of dimeric (band A; 9% band intensity) and monomeric (band B; 91% band intensity) forms. The band intensity profile of this lane was similar to SDS-PAGE analysis. Thus, the selective immunodetection of γ-conglutin by its antibody confirmed the presence of γ-conglutin in the elution fraction. Similar to SDS-PAGE profile, reduced γ-conglutin sample (Lane 5) exhibited non-cleaved γ-conglutin at 50 KDa (band D) and heavy subunit at 33 KDa (band E).

The HPLC chromatogram of purified γ-conglutin fraction (Fig. 1E) showed the presence of two peaks – an impurity peak A at 26.19 min and γ-conglutin peak B at 29.53 min. The percent AUC i.e. % purity of γ-conglutin with respect to total proteins (peak B) eluting at 29.53 min was found to be of 95.2%. Purified fractions of γ-conglutin were analysed by mass spectrometry (LC-MS/MS) to substantiate identity as γ-conglutin. The MS/MS spectra of peptides were analysed using Mascot sequence matching software (Matrix Science) with MSPnr100 database. A summary of all hit proteins with their accession number was obtained (Table 1). For each protein match, the overall protein score was calculated. A higher protein score indicated a more confident match. Protein scores >67 (threshold score) were considered to be significant ($p < 0.05$). We found that γ-conglutin protein from *L. angustifolius* (accession number - Q42369) hit was the highest score of 2137. This high score indicated a low probability for the observed match to be a random event (Cottrell, 2011). In Q42369, 12 peptide sequences matched with γ-conglutin protein database contributing to 35% coverage of the protein sequence (Supplementary Fig. S1). The second highest score (OIW19056.1) and other protein scores (OIW08851.1, OIV97544.1, B9RG92, OIW00888.1) in Table 1 did not present a significant number of peptide sequences required for identification of the proteins. (Carr et al., 2004) Also, the molecular weight and pI of the proteins (except Q42369) listed in Table 1 did not match with either molecular weight (48 KDa) or pI (7–8) of γ-conglutin (Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008) As a result, the identity of the purified protein was concluded to be γ-conglutin.

### 3.2. Proteolytic hydrolysis of γ-conglutin protein

Peptides and amino acids generated from hydrolysis of γ-conglutin were evaluated for their potential use as an oral antidiabetic agent. The *in-vitro* gastrointestinal proteolytic hydrolysis was carried out in two steps, first by pepsin and then by pancreatin as described in Fig. 2A. The concentration of purified γ-conglutin (before hydrolysis) was 4.8 ± 0.61 mg/ml. The DH was determined by calculating free amino acids generated by pepsin and pancreatin enzymes during hydrolysis (Fig. 2B). The γ-conglutin hydrolysis stabilized around DH of 3.5 to 4% after the first 30 min of pepsin hydrolysis. In the second step, DH increased to 18.3% after 450 min in the presence of pancreatin.

The SDS-PAGE profile of the hydrolysate after every 60 min of hydrolysis is shown in Fig. 2C. It was observed that pepsin majorly hydrolysed γ-conglutin into polypeptides with a molecular weight ≤ 30 KDa, while pancreatin hydrolysed large peptides into small peptides (≤6.5 KDa) and amino acids except peptides around 15 KDa. Complete profiling of peptides in γ-conglutin hydrolysate was obtained from MS/MS analysis (Supplementary Table 1). A total of 32 peptides were detected and sequenced, having molecular weights in range of 704.4 Da to 3117.7 Da.

**Table 1**
Summary of all the hit proteins generated by MS/MS ion search with matching peptide sequences obtained from tandem mass spectrometric analysis of purified γ-conglutin.

| Rank order | Accession | Score | Mol. Wt. (Da) | pI | Coverage (%) | Matches | Sequences | Peptides sequence |
|---|---|---|---|---|---|---|---|---|
| **1** | **Q42369** | **2137** | **48,885** | **7.66** | **35** | **88 (65)** | **12 (10)** | **HLVIPTK VGFNSNSLK ISGGAPSVDLILDK QGEYFIQVNAIR KISGGAPSVDLILDK TPLMQVPLLLDLNGK RTPLMQVPLLLDLNGK ISGGAPSVDLILDKNDAVWR AGIALGAHHLEENLVVFDLER YSTSNGAILFGDINDPNNNNYIHNSLDVLHDLVYTPLTISK** |
| 2 | OIW19056.1 | 699 | 11,712 | 9.38 | 17 | 19 (18) | 1 (1) | AAAANTPGLNPSNAGSLPGK |
| 3 | OIW13331.1 | 209 | 12,957 | 9.49 | 25 | 6 (5) | 3 (2) | YGAGGNYY ETAANIGASAK MNQAELDKLAAR |
| 4 | OIW13848.1 | 201 | 11,872 | 9.07 | 50 | 10 (10) | 5 (5) | LVLVASSK KLTSFELA GESQVVSGTNYR EIADFAVTEHNK NYQAVVYEKPWLHFK |
| 5 | OIW15434.1 | 195 | 46,671 | 9.14 | 22 | 8 (7) | 7 (6) | SIVPIASGR VLYDVPNSR LSEPAYVAVR QIIQSPTYIVR SSLYYVNLFAIR TTGTSTPPQGLLGLGR IVNIPPPALAFNPTTGAGTIFDSGTVFTR |
| 6 | OIW08851.1 | 127 | 13,967 | 5.37 | 11 | 2 (2) | 1 (1) | AVDVTGPDGANVQGSR |
| 7 | A0A061FK97 | 122 | 12,375 | 9.34 | 46 | 7 (2) | 5 (2) | ADLIAYLK NMAVNWEEK QGPNLNGLFGR TLYDYLLNPK QSGTTPGYSYSAANK LSGTTPGYSYSAANK |
| 8 | OIV97544.1 | 97 | 24,667 | 8.79 | 7 | 1 (1) | 1 (1) | DQYGNPIQLTDQYGNPVK |
| 9 | OIV98539.1 | 97 | 45,201 | 6.72 | 17 | 6 (5) | 6 (5) | VAVVLLNR MYVLKPIA ETADALVSTGLSK TFASWGIDYLK NSITANWDDIDIPTK TTGDINDSWESMITR |
| 10 | OIV89537.1 | 77 | 13,479 | 9 | 24 | 2 (2) | 2 (2) | VFASGNDQIR AGDLLIFNYDSTSHNVVAVDR |
| 11 | B9RG92 | 74 | 46,284 | 9.46 | 2 | 1 (1) | 1 (1) | SSLYYVNLMAIR |
| 12 | OIW00888.1 | 73 | 11,670 | 9.26 | 10 | 1 (1) | 1 (1) | ALVAAAQSTADK |
| 13 | OIV96297.1 | 72 | 60,824 | 9.27 | 8 | 3 (2) | 3 (2) | QYLGQQFYLR DQIGSFYYFPSLGFHK IPVPFPDPAGDYTILIGDWYK |
| 14 | OIW10115.1 | 68 | 19,175 | 9.16 | 33 | 4 (2) | 3 (2) | GPLGLVEQPPSR QLNLGLPVATVYFNSQK VTLTGNMDNLYTLVMTDPDAPSPSEPSHR |

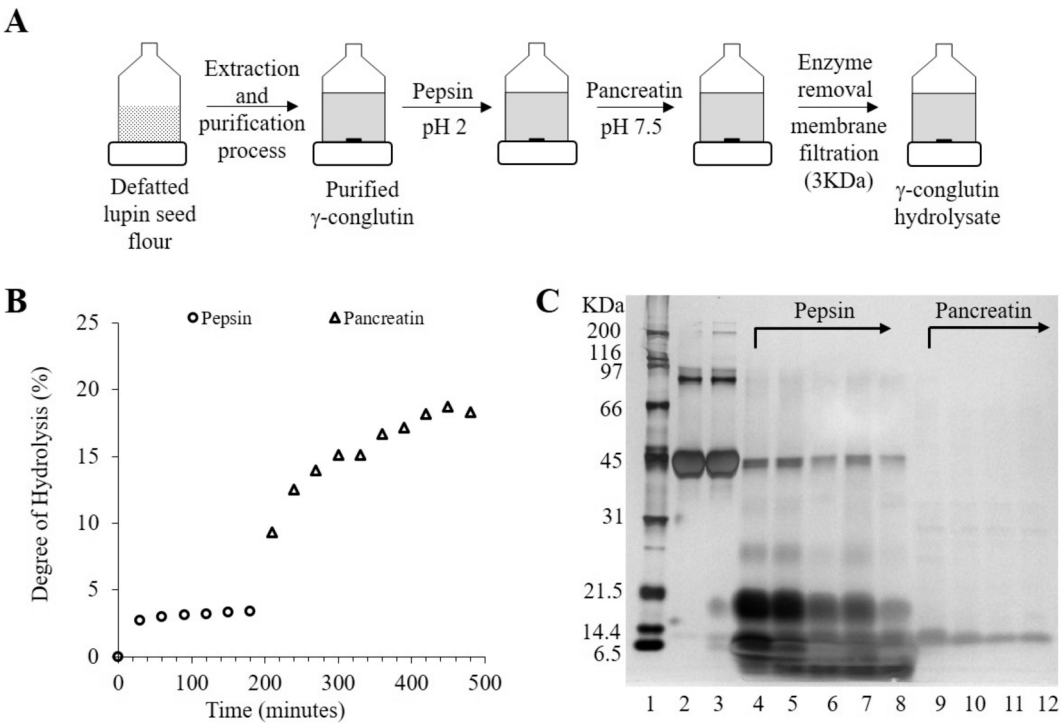


**Fig. 2. Proteolytic hydrolysis of γ-conglutin:** (A) Two stage gastrointestinal proteolytic digestive system; (B) Degree of hydrolysis of γ-conglutin by pepsin and pancreatin enzyme at 30 min intervals from starting point (C) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of γ-conglutin at different time hydrolysis intervals: γ-conglutin (lane 2); γ-conglutin at pH 2 before addition of pepsin (lane 3); hydrolysate after 30, 60, 90, 120 and 180 min of pepsin addition (lane 4, 5, 6, 7 and 8 respectively); hydrolysate after 30, 60, 120 and 240 min of pancreatin addition (lane 9, 10, 11 and 12 respectively).

### *3.3. γ-conglutin peptides do not have insulinotropic action in pancreatic β cells*

The insulinotropic action of lupin extract hydrolysate has been previously reported (Tapadia et al., 2019). In the present study, the objective was to determine if γ-conglutin in the extract was responsible for this effect. At first, cell viabilities of BRIN-BD11 and INS-1E cells in the presence of γ-conglutin peptides were studied to determine if peptides resulted in any release of insulin by cell death. The peptides did not have any detrimental effects on the cell viability (Fig. 3A and 3B). Acute (20 min) insulin secretion from both BRIN-BD11 and INS-1E cells at 16.7 mM glucose in presence of different peptide concentrations of γ-conglutin hydrolysate (200–20 μg/ml) showed no difference as compared to control (Fig. 3C and 3D). Moreover, these secretion values were significantly lower than the positive controls i.e. alanine (10 mM) and lupin extract hydrolysate (200 μg/ml) (Tapadia et al., 2019). Thus, γ-conglutin peptides did not exhibit direct insulinotropic action in either of the pancreatic β cell models.

### *3.4. γ-conglutin peptides possess strong insulin-mimetic actions*

*In vitro* differentiated primary HSMM were used to study the insulin-mimetic action of γ-conglutin peptides. First, we investigated if phosphorylation events associated with insulin signalling were elicited directly by γ-conglutin peptides. Then, the two major cellular outputs of insulin signalling, glucose uptake and protein synthesis, were assessed in response to γ-conglutin peptides supplementation.

Acute exposure of differentiated HSMM to γ-conglutin peptides (200 μg/ml) resulted in 2-fold increase in phosphorylation levels of mTOR (Fig. 4A) and AKT (Fig. 4B) compared to control. This indicated that γ-conglutin peptide, like insulin, activated the central mTOR/AKT signalling pathway. Furthermore, increases in downstream signalling proteins GSK3β (~1.4 fold; Fig. 4C), 70S6K (~3.6 fold; Fig. 4D) and S6 (~2.5 fold; Fig. 4E) were observed. Also, γ-conglutin peptides induced phosphorylation of ERK 1/2 (1.7 fold; Fig. 4F) similar to the levels stimulated by insulin.

Since γ-conglutin peptides activated mTOR/AKT/GSK3β signalling pathway, next we assessed their effects on cellular glucose uptake and glycogen synthesis. Myotubes were treated with insulin (100 nM) or γ-conglutin peptides (200 μg/ml) and glucose uptake in myotubes was determined. Similar to insulin, γ-conglutin peptides promoted cellular glucose uptake (~1.4 fold) (Fig. 5A). Glycogen content in myotubes was evaluated by quantifying glucose generated from acid mediated glycogenolysis, as detailed in Materials and Methods 2.14. A significant increase in glycogen content (~2 fold; Fig. 5B) was observed in cells treated with γ-conglutin peptides, indicating that the peptides stimulated skeletal muscle glycogen synthesis.

Increased phosphorylation levels of mTOR downstream effectors – 70S6K and S6 suggested that incubation of myotubes with γ-conglutin peptides triggered the mTOR/70S6K/S6 signalling axis, responsible for the control of cellular protein synthesis. Thus, we next assessed nascent protein synthesis in the presence of the peptides. Fig. 6A displays representative images captured at two wavelengths to detect nascent proteins (561 nm, AlexaFluor®) and nuclei (402 nm; HCS Nuclear Mask® blue stain). Nascent proteins were quantified as the integrated density of pixel per unit area (mean) and reported as fold change to control unstimulated cells (Fig. 6B). Insulin, γ-conglutin peptides at 200 μg/ml and 20 μg/ml all exhibited an increase in protein synthesis (~1.56, ~1.55, and ~1.34 fold, respectively) compared to control. Cycloheximide (10 μM), an inhibitor of translation elongation process, effectively blocked protein synthesis (~0.6 fold) compared to control. Altogether, these data demonstrate that γ-conglutin peptides can directly stimulate insulin signalling associated pathways, ultimately promoting glucose update, glycogen and protein synthesis in primary HSMM.

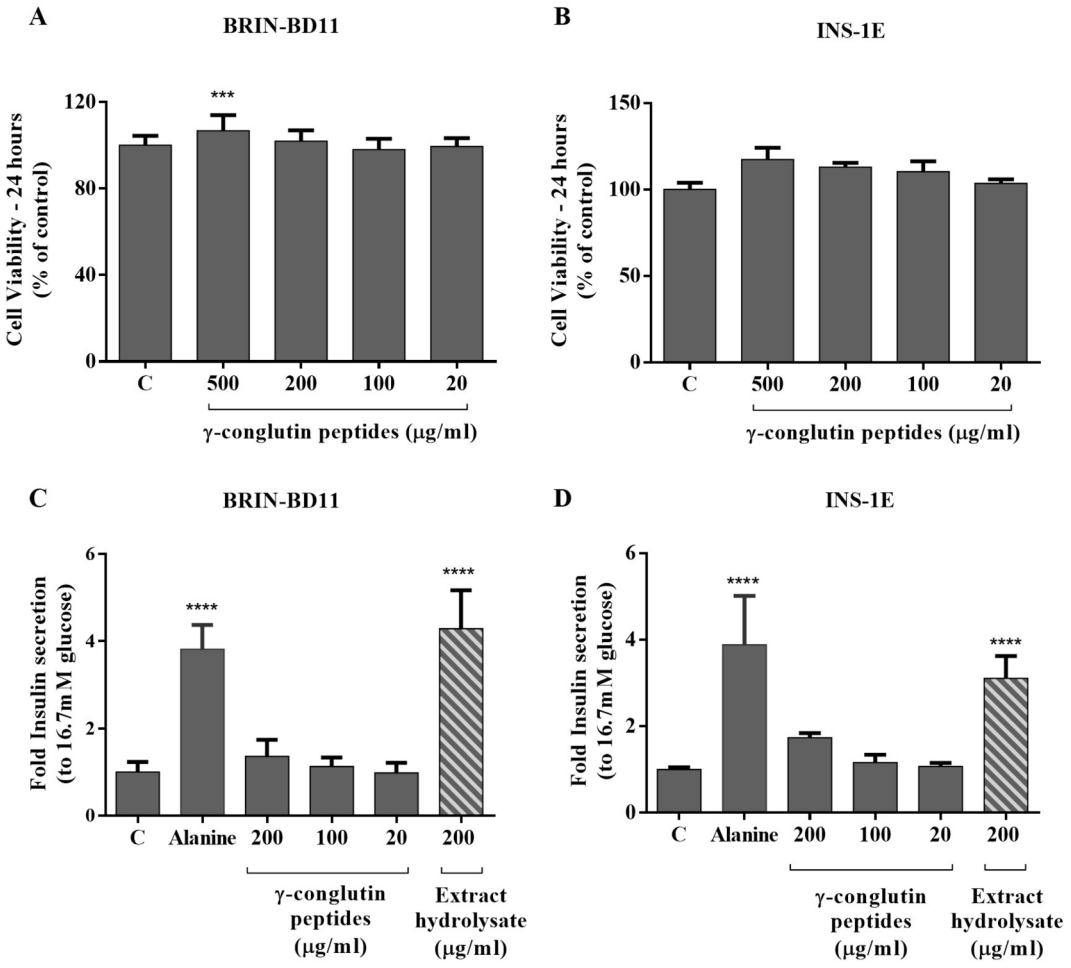


**Fig. 3. γ-conglutin peptides do not exhibit insulinotropic action in pancreatic β cells:** (A) BRIN-BD11 and (B) INS-1E cell viability at different γ-conglutin peptide concentrations (500–20 μg/ml) over 24 h. Cell viability is reported as a percentage of control 'C'. Dose-dependent effect of γ-conglutin peptides in 16.7 mM glucose on acute (20 min) insulin secretion from (C) BRIN-BD11 and (D) INS-1E cells. The response was compared with control (16.7 mM glucose) referred as 'C', positive control alanine (10 mM L-alanine in 16.7 mM glucose) and lupin extract hydrolysate (200 μg/ml in 16.7 mM glucose). Insulin secretion is reported as values normalised with control. Values are mean ± standard deviation of three independent experiments; ****$p \leq 0.0001$ and ***$p \leq 0.001$ are significantly different as compared to control.

### *3.5. γ-conglutin peptides inhibited DPP4, but not α-glucosidase activity*

The γ-conglutin peptides were investigated for their potential DPP4 inhibitory activity using an established *in-vitro* biochemical screening assay kit. As observed in Fig. 7A, positive control sitagliptin at 100 μM (manufacturer recommended concentration) completely inhibited DPP4 activity. The γ-conglutin protein (non-hydrolysed) did not exhibit detectable inhibitory action, whereas its hydrolysed form (γ-conglutin peptides) inhibited the enzyme in a dose-dependent manner. This indicated that the intact protein had to be digested by pepsin and pancreatin to produce DPP4 inhibitory peptides. To rule out the possibility that the observed inhibition could be the result of a random mix of peptides competing with the substrate for the enzyme's catalytic site. A peptide digest (500 μg/ml) obtained from ThermoFisher Scientific, was used as a negative control, and presented no inhibitory action.

On the other hand, when γ-conglutin intact protein and peptides ranging from 100 μg/ml to 1000 μg/ml were tested against α-glucosidase activity, only minimal reduction in enzyme activity (6%) was observed (Fig. 7B). The inhibitory action of a positive control, acarbose, was evaluated at different concentrations (0.001–25 mM) in the optimized assay condition (Supplementary Fig. S2). As indicated in Fig. 7B, acarbose at 10 mM completely inhibited α-glucosidase activity.

## 4. Discussion

In the present study, different cellular and molecular mechanisms responsible for the glucose lowering property of γ-conglutin were investigated. Initially, the purified γ-conglutin was characterized, then hydrolysed using digestive enzymes to recreate *in-vitro* gastrointestinal digestive conditions, allowing us to study the digested products in human skeletal muscle and β cell models. Peptides from γ-conglutin hydrolysis were evaluated for their insulinotropic action in pancreatic β cells (BRIN-BD11 and INS-1E), insulin-mimetic action in primary HSMM, and last, for their inhibitory potential against the enzymes DPP4 and α- glucosidase. We found that γ-conglutin peptides do not exhibit insulinotropic action in pancreatic β cells, suggesting that different constituents in the earlier studied lupin protein extract (Tapadia et al., 2019) possess this activity. On the other hand, γ-conglutin peptides activated insulin signalling associated pathways responsible for glycogen and protein synthesis, exhibiting insulin-mimetic action in HSMM cells. The peptides increased glucose transport into the cells, and also activated mTOR/p70S6K signalling, a key regulator of cellular protein synthesis. In addition, the peptides exhibited strong DPP4 inhibitory activity, highlighting yet another mechanism by which γ-conglutin can help modulate glucose homeostasis.

The beneficial effects of lupins for blood glucose control, particularly its γ-conglutin, have been documented in the literature. Pasta enriched

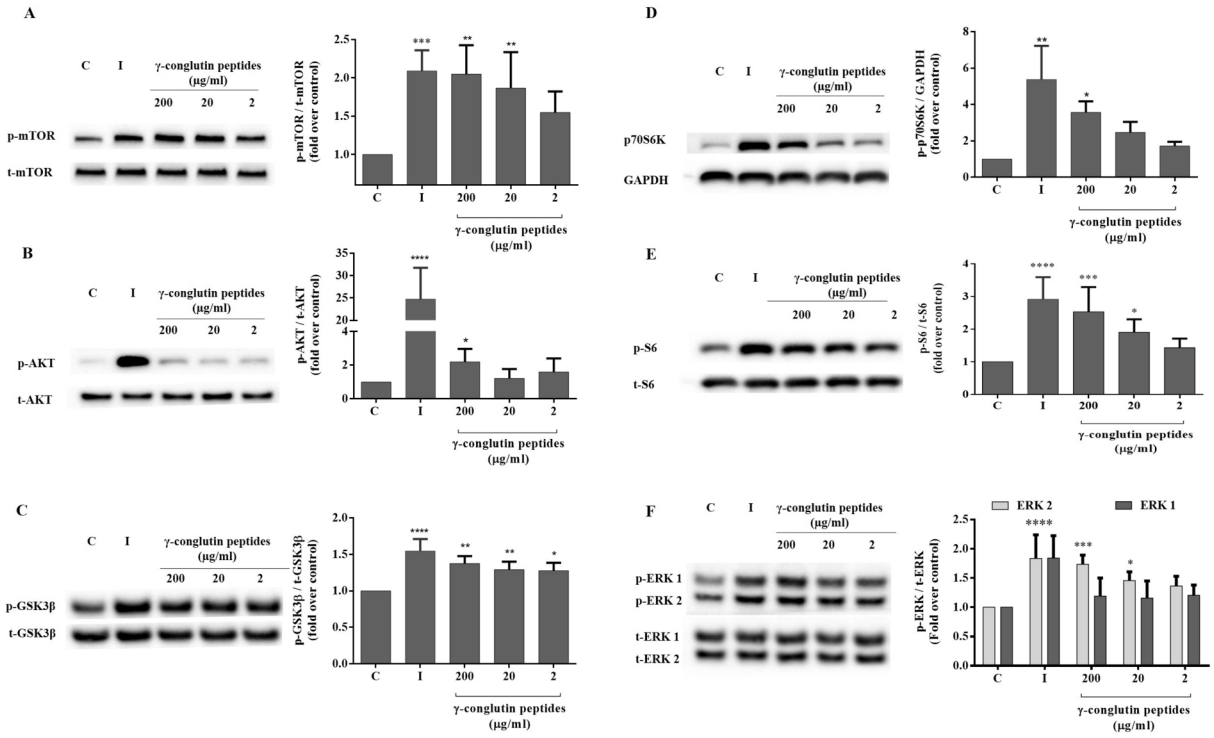


**Fig. 4. γ-conglutin peptides stimulate mTOR downstream pathway in HSMM:** Western blot analysis of (A) mammalian target of rapamycin (mTOR) and (B) AKT (protein kinase B) (C) glycogen synthase kinase-3β (GSK3β) (D) 70 KDa ribosomal protein S6 kinase (70S6K) (E) ribosomal protein S6 (S6) and (F) extracellular signal-regulated kinase (ERK1/2) in HSMM cell lysate and ratio of their band intensities (phosphorylated (p)/total (t) or phosphorylated (p)/GAPDH) in presence of vehicle (control referred to as 'C'), positive control – insulin (100 nM) (referred as 'I') or γ-conglutin peptides (200–2 μg/mL). The ratio of band intensities is reported as values normalised with control. Values are mean ± standard deviation of three or more independent experiments; ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$ and *$p \leq 0.05$ are significantly different as compared to control. Immunoblots shown are representative of three or more independent experiments.

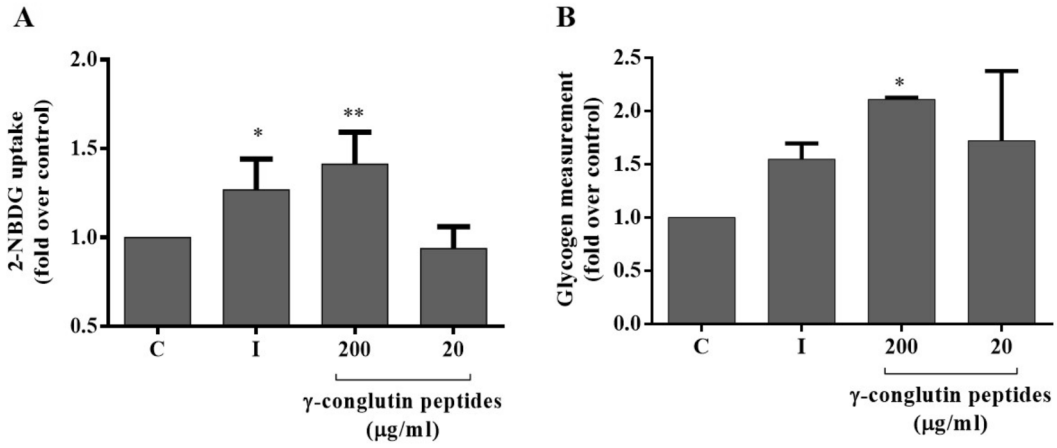


**Fig. 5. Peptides increase cellular glucose uptake and glycogen synthesis in HSMM:** (A) 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose (2-NBDG) uptake and (B) Glycogen content in myotubes (HSMM) in presence of vehicle (control referred as 'C'), insulin (100 nM) (referred as 'I'), and γ-conglutin peptides (200–20 μg/ml). The response is reported as values normalised with control. Values are mean ± standard deviation of three or more independent experiments; ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$ and *$p \leq 0.05$ are significantly different as compared to control.

with γ-conglutin significantly reduced blood glucose in rats after a glucose overload compared to pasta with other lupin isolates (α + β + δ conglutins), or ovalbumin control (Capraro et al., 2014). In another report, a dose-dependent reduction of blood glucose levels was observed on acute oral administration of purified γ-conglutin in rats (Magni et al., 2004). Similar acute hypoglycaemic effect was also detected in healthy mouse models and human volunteers administered with γ-conglutin enriched preparations (Bertoglio et al., 2011). Chronic treatment of γ-conglutin (in D-glucose drinking water – 10%) in rats for 3 weeks resulted in reduced plasma glucose and insulin levels and attenuated the increased insulin resistance index compared to control (D-glucose alone). Here, γ-conglutin administration counteracted the hyperglycaemic conditions and improved insulin sensitivity which otherwise was decreased by D-glucose water (Rosa Lovati et al., 2012). Administration of γ-conglutin for one week to neonatal streptozotocin-induced diabetic rats decreased glucose and increased insulin levels, which was paralleled by augmented insulin content in pancreatic β cells (Vargas-Guerrero et al., 2014). Altogether, these studies highlight the potential of γ-conglutin as an antidiabetic agent to manage diabetic patients.

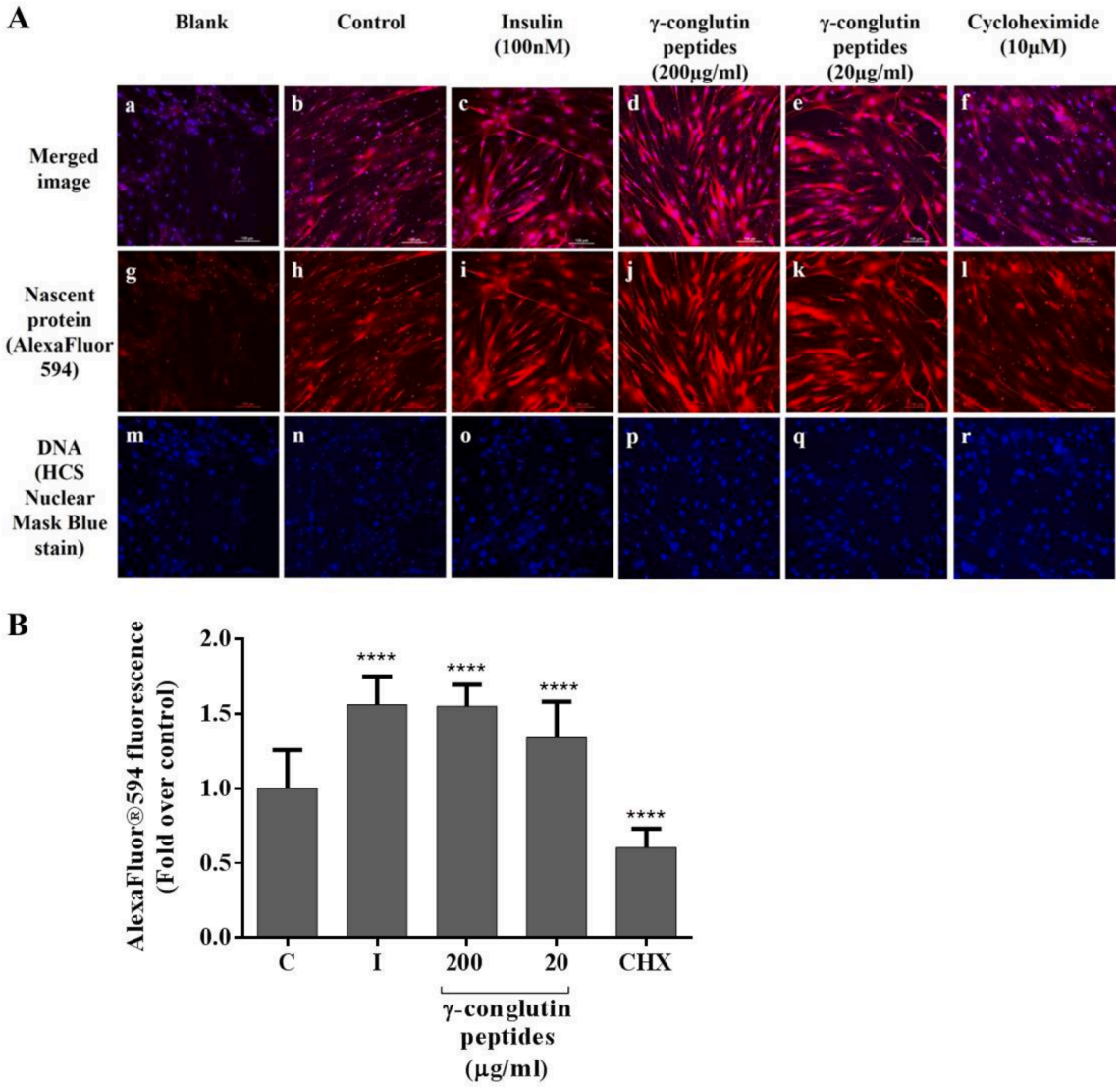


**Fig. 6. Peptides stimulate protein synthesis in HSMM:** Confocal laser scanning microscopy images of nascent proteins in cells co-cultured with o-propargyl-puromycin (OPP) and different treatments. 'Blank' represents cells unlabelled with OPP, 'C' (control) refers the OPP treatment only, and Insulin (I) 100 nM, γ-conglutin peptides (200 μg/ml and 20 μg/ml), cycloheximide (CHX) 10 μM refers to OPP labelled cells with the respective treatments. The cells are stained with AlexaFlour594 (nascent protein) and HCS NuclearMask® Blue stain (DNA). Images shown are representative of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We have previously developed an extraction and purification method to isolate γ-conglutin from lupin to investigate its mechanisms of action (Mane et al., 2017, 2018; S. Mane et al., 2014; Tapadia et al., 2019). Here we show the identity of γ-conglutin in eluted fractions was found to be ~100% based on semi-quantitative SDS-PAGE analysis, and 95.2% based on quantitative RP-HPLC analysis. In general, digested dietary protein hydrolysates are more readily bioavailable for target action as compared to their undigested intact polypeptide forms (Gaudel et al., 2013; Geerts et al., 2011; Koopman et al., 2009; Power, Hallihan, & Jakeman, 2009). Therefore, we developed an *in vitro* proteolytic model using pepsin and pancreatin to digest the purified protein in a gastro-intestinal digestive mimetic manner. This has allowed us to directly assess the digested products in cultured cells and gain further insight into the cellular mechanisms of action of γ-conglutin digestion products.

Previous work from our laboratory shows that hydrolysates generated from lupin protein extract exhibited insulinotropic action by enhancing glucose metabolism and stimulating a Gαq/PLC/PKC pathway in pancreatic β cells (Tapadia et al., 2019). However, the component responsible for this action was not determined. We hypothesised that γ-conglutin, was the protein that possessed insulinotropic action in the hydrolysates. Therefore, in the present study we generated peptides by the hydrolysis of pure γ-conglutin. Surprisingly, these peptides displayed no detectable insulinotropic properties. This indicates that biomolecules such as proteins (albumins, other globulins), carbohydrates, alkaloids, carotenoids, polyphenolics, tocopherols, others than γ-conglutin might be responsible for the potent insulinotropic action previously observed. Further studies are necessary to identify and characterise such insulinotropic component the lupin extract.

Skeletal muscle is the major glucose homeostasis regulator in the body, mainly due to its large mass. Insulin is a key factor required for the transfer, metabolism and storage of blood glucose into muscle cells (Buczkowska & Jarosz-Chobot, 2001). It promotes glucose uptake, increases the rate of glycolysis, and stimulates glycogen synthesis. Also, insulin increases the transport of amino acids across the cell membrane and triggers cell signalling events leading to activation of protein synthesis (Dimitriadis, Mitrou, Lambadiari, Maratou, & Raptis, 2011). Binding of insulin to the extracellular α subunit of the insulin receptor

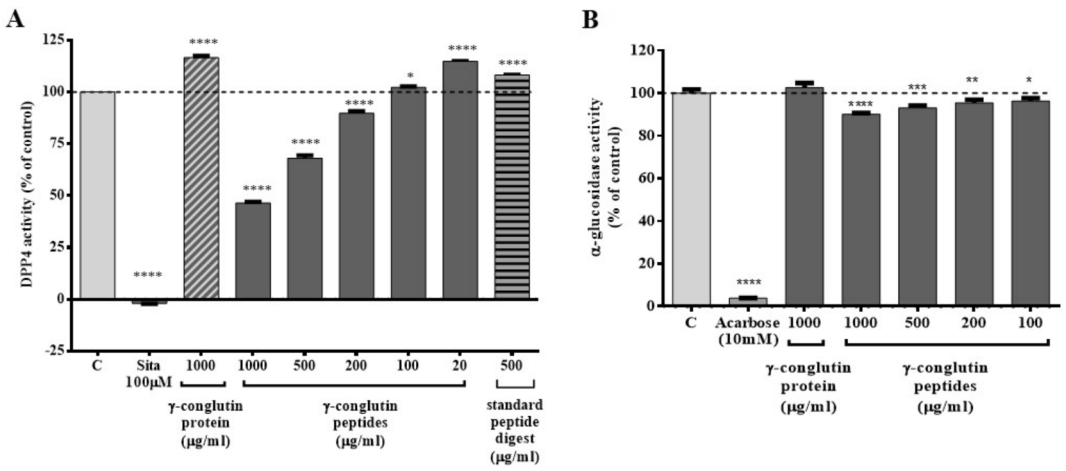


**Fig. 7. Effect of peptides on DPP4 and α-glucosidase enzyme activity:** (A) effect of positive control - sitagliptan (100 μM) (sita), γ-conglutin protein, peptides (1000–20 μg/ml), and negative control (commercial peptide digest, 500 μg/ml) on the enzymatic activity of dipeptidyl peptidase 4 (DPP4) compared to control – 'C'. (B) Effect of positive control (acarbose, 10 mM), γ conglutin protein and peptides on the enzymatic activity of α-glucosidase compared to control 'C'. Values are mean ± standard deviation of three independent experiments; ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$ and *$p \leq 0.05$ are significantly different as compared to control.

(IR) results in activation of tyrosine kinase present on each intracellular β subunit of IR. Auto phosphorylation of tyrosine residues triggers the phosphorylation of insulin receptor substrate (IRS-1). The activated IRS-1 serves as the binding site for various signalling proteins, the most important being phosphoinositide 3-kinase (PI3K), which subsequently activates 3-phosphoinositide-dependent protein kinase 1 (PDK-1) (Boucher, Kleinridders, & Kahn, 2014). Further, PDK-1 phosphorylates and activates downstream AKT and PKC cascades. Phosphorylation of AKT at Ser-473 residue by mTOR complex 2 (mTORC2) is also required for its complete activation. PDK-1 and mTORC2 mediated AKT phosphorylation trigger activation of multiple downstream targets involved in regulating protein, lipid and glycogen synthesis, translocation of glucose transporters to the cell surface, ultimately controlling cell metabolism, cycle and survival (Mackenzie & Elliott, 2014).

At first, we investigated if γ-conglutin peptides were able to activate downstream proteins involved in insulin signalling pathway in HSMM. We observed that γ-conglutin peptides induced phosphorylation of mTOR and AKT. Similar results have been reported in C2C12 mouse myoblasts, however, the end effect of glucose transport into the cells was not investigated (Terruzzi et al., 2011). In the present study, we showed that HSMM cells uptake significantly more glucose when stimulated with γ-conglutin peptides as compared to insulin (100 nM). Similarly, γ-conglutin has been previously demonstrated to increase glucose consumption in HepG2 hepatocytes and 3T3-L1 adipocytes when exposed to high glucose concentration medium (Muñoz et al., 2018). However, in a previous study normal glucose concentrations did not significantly increase glucose consumption in HepG2 cells (Rosa Lovati et al., 2012).

Insulin regulates glycogen synthesis via AKT phosphorylation of GSK3β at serine residues that block and prevent its action. The inactivation of GSK3β leads to subsequent dephosphorylation and activation of glycogen synthase (GS) that catalyses the addition of glucose monomers onto growing glycogen chains (Summers et al., 1999). We observed that γ-conglutin peptides increased phosphorylation of GSK3β, subsequently leading to an increase in glycogen content. Altogether, the dataset presented herein shows that γ-conglutin peptides alone are able to stimulate the mTOR/AKT/GSK3β signalling axis, leading to an increase in glucose uptake and glycogen content, key factors responsible for maintaining blood glucose levels in humans.

For the first time, we show that γ-conglutin peptides can increase skeletal muscle protein synthesis in primary human myotubes. AKT regulates protein synthesis by inactivation of tuberous sclerosis complex (TSC1/2), a negative regulator of mTORC1, subsequently activating mTORC1 downstream proteins (Yoon, 2017). Further, mTORC1 phosphorylates its effectors eukaryotic initiation factor 4E binding protein 1 (4EBPI) and 70S6K which together promote the biosynthesis of S6 and cell cycle regulatory proteins responsible for initiating translation (Shimobayashi & Hall, 2014). We observed that γ-conglutin peptides induced phosphorylation of 70S6K and S6, demonstrating that the protein synthesis arm of AKT signalling can be directly activated by these peptides. These results are in agreement with a previous study reporting similar phosphorylation and stimulation of 70S6K and eukaryotic initiation factor 4E (eIF4E) in C2C12 myoblasts cultured with γ-conglutin protein (Terruzzi et al., 2011). Furthermore, in the present study, an increase in phosphorylated levels of ERK1/2 was observed. ERK phosphorylates downstream kinases that lead to activation of eIF4E, a rate-limiting step in protein synthesis initiation. This represents another potential mechanism that enhances protein synthesis in skeletal muscle cells in response to stimulation by γ-conglutin peptides.

Glucagon like peptide 1 (GLP-1) and gastric inhibitory peptide (GIP), secreted by small intestinal L cells and K cells respectively, stimulate insulin synthesis from pancreatic β cells (Drucker & Nauck, 2006; Lynn et al., 2001; Nauck, Baller, & Meier, 2004). These incretin hormones are rapidly degraded by DPP4 in plasma. Synthetic DPP4 inhibitors, such as sitagliptin and saxagliptin, suppress the activity of DPP4, increasing the half-life of circulating incretins in circulation, and are utilised as oral antidiabetic agents. Food derived proteins have previously been isolated, hydrolysed and characterised to evaluate their DPP4 inhibitory properties (Power, Nongonierma, Jakeman, & FitzGerald, 2014) with lupin and soy peptides previously being reported to be inhibitory (Lammi et al., 2018). Munoz et al. have previously shown that γ-conglutin peptides at high concentration (5 mg/ml) completely blocked DPP4 (Muñoz et al., 2018). However, in that study, the SDS-PAGE profile of the purified γ-conglutin displayed a range of different protein impurity bands between 56 and 100 KDa and 56–21 KDa and the purity of γ-conglutin protein was not reported. Therefore, peptides generated from co-purified proteins might have also contributed to the DPP4 inhibitory action. Here, we add to these findings and demonstrate that pure γ-conglutin peptides present concentration-dependent DPP4 inhibitory capacity, demonstrating significant inhibition with doses as little as 200 μg/mL. Absence of DPP4 inhibitory effects by intact γ-conglutin indicate that the protein needs to be digested to generate inhibitory bioactive peptides.

The *in-vitro* biochemical screening assays we have used in this study do not account for the influence of several factors such as bioavailability and stability of peptides, the interaction of peptides with serum components, the effect of serum proteases and fluid dynamics in the

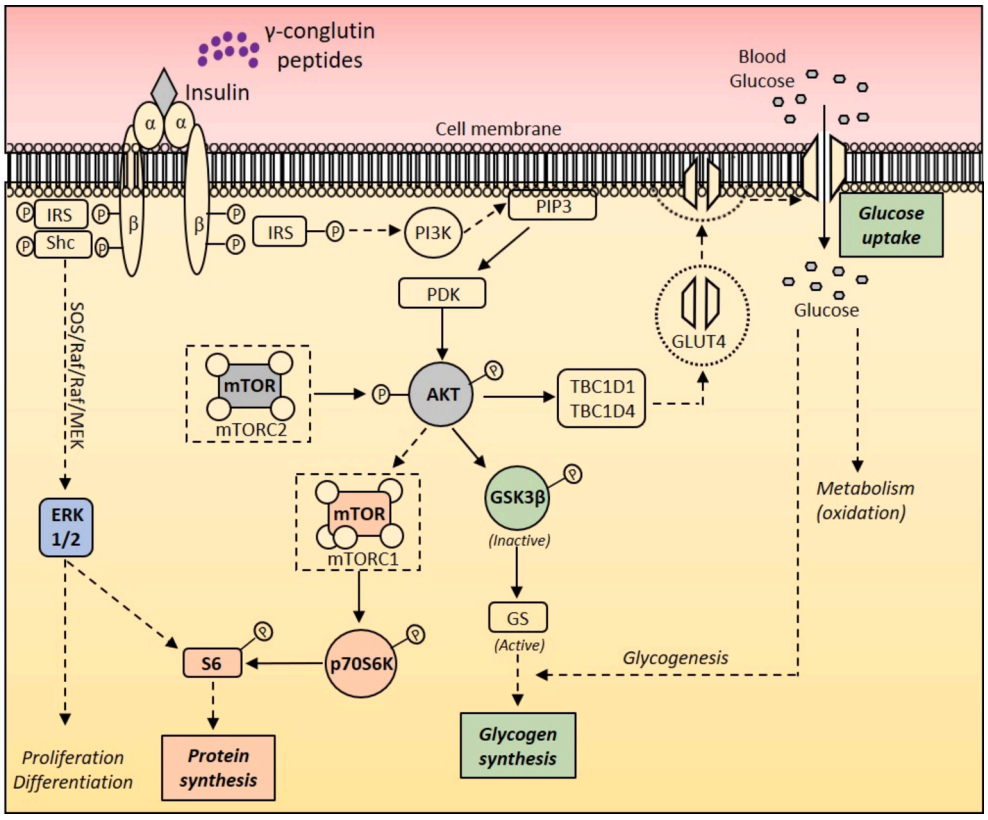


**Fig. 8. Activation of insulin signalling pathway by γ-conglutin peptides:** γ-conglutin peptides, like insulin, activates mTOR/AKT pathway via activation of PI3K/PDK and mTORC2. Activated Akt is the central mediator responsible for regulating insulin's metabolic effects, glycogen and protein synthesis, and glucose transport into the cells. Akt mediates protein synthesis by activating mTORC1/p70S6K/S6 pathway. Also, Akt directly activates GSK3β/GS pathway leading to glycogen synthesis. AKT mediates GLUT-4 translocation to the cell membrane consequently causing an increase in cellular glucose uptake. The peptides also affect mitogenic pathway (Ras/Raf/MAPK) by activating ERK1/2 responsible for cellular growth, differentiation and protein synthesis. Thus, γ-conglutin exhibits insulin-mimetic behaviour. IRS: insulin receptor substrate; PI3K: phosphoinositide-3-kinase; PIP3: phosphatidylinositol (3,4,5)-triphosphate; PDK: 3-phosphoinositide-dependent protein kinase 1; mTORC: mammalian target of rapamycin complex; Akt: protein kinase B; GSK3 β: glycogen synthase kinase 3 β; GS: glycogen synthase; GLUT-4: glucose transporter 4; p70S6K: 70 KDa ribosomal protein S6 kinase; S6: ribosomal protein S6; ERK: extracellular signal-regulated kinase; TBC1D1/4: TBC1 domain family member 1/4.

inhibition kinetics. Thus, future *in vivo* studies confirming our observations are required to validate the potential efficacy of pure γ-conglutin for blood glucose regulation.

The insulin-mimetic and DPP4 inhibitory action of gamma conglutin hydrolysate is quantitatively similar to different naturally derived protein hydrolysates. Whey protein hydrolysate (WPH) has been reported to exhibit dose dependent insulinotropic property in pancreatic beta cells. Insulin secretion in BRIN-BD11 cells was significantly higher with whey protein hydrolysate than whey protein. Insulinotropic action of whey protein hydrolysate (100ug/ml) and lupin extract hydrolysate (200ug/ml) were 3 fold and 4 fold higher than control respectively (Nongonierma et al., 2013). Egg white hydrolysate (5 mg/ml) alone has been reported to increase ERK1/2 phosphorylation (1.6 fold) in 3T3-F442A preadipocytes when incubated for 48 h compared to untreated cells. Moreover, this effect was blocked when the cells were incubated with insulin receptor antagonist (S961), indicating insulin mimetic effect of egg white hydrolysate is mediated through insulin signaling (Jahandideh, Chakrabarti, Davidge, & Wu, 2017). In the present dataset, γ-conglutin peptides (200ug/ml) induced ERK phosphorylation (~1.7 fold), similar to levels stimulated by insulin (100 nM). Gamma conglutin hydrolysate (1 mg/ml) potently inhibited DPP4 in a similar range as other peptides obtained from salmon skin gelatin (IC50: 1.35 mg/ml), whey protein hydrolysate (IC50: 1.34 mg/ml) and rice protein hydrolysate (IC50: 1.45 mg/ml) (Hatanaka, Uraji, Fujita, & Kawakami, 2015; Li-Chan, Hunag, Jao, Ho, & Hsu, 2012; Nongonierma & FitzGerald, 2013).

## 5. Conclusion

The present study advances our understanding of the cellular and molecular mechanisms underlying the glucose lowering effects of the lupin protein γ-conglutin. Operating as an insulin mimetic, γ-conglutin peptides induced phosphorylation and activation of AKT and several of its downstream targets responsible for a range of metabolic processes (Fig. 8). Increased glucose uptake, glycogen and protein synthesis were observed in primary HSMM cells in response to γ-conglutin peptides. Significant inhibitory activity against DPP4 constitutes another mechanism of action of γ-conglutin peptides, highlighting the multi-mode potential characteristics of this protein should it be translated to an option for treatment of type 2 diabetes patients. In contrast to previously hypothesised, γ-conglutin peptides did not elicit an insulinotropic effect in β-cells, therefore the identity of the component showing this activity previously for hydrolysate of lupin protein extracts remains to be determined. The results presented herein offer a rationale to expand the current market of lupin from primarily stockfeed, towards value-added lupin-based health-functional food products for human consumption as well as the inclusion of lupin proteins in nutraceuticals and future antidiabetic formulations.

## Ethical statement

We declare that all the data presented in the manuscript were produced by the Authors partnership and used with the permission from all Authors.

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## Author contributions

M.T., S.J., R.U., P.N. and R.C. designed the study; M.T. and R.C. performed experiments; M.T. and R.C. analysed data and wrote the paper; M.T. and R.C. are the guarantors of this study and take full responsibility for the work, including the study design, access to data, and the decision to submit and publish the manuscript. All authors reviewed and approved the final manuscript.

## Declaration of Competing Interest

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

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2021.104786.

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