



Original Article

Expression, purification and biological characterisation of recombinant human irisin (12.5 kDa)

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ABSTRACT

Fibronectin type III domain containing 5 (FNDC5) is a transmembrane protein. Upon cleavage, it yields a peptide called irisin that is supposedly bind to an unknown receptor and facilitates browning of white adipose tissue (WAT). Increased levels of irisin are associated with increased levels of energy expenditure markers PGC-1 α , UCP-1, besides abundance of beige adipocytes in WAT. Though varied sizes of irisin were reported in humans and rodents it is not yet clear about the actual size of the irisin produced physiologically. Hence, we cloned and expressed human irisin (32–143 aa of FNDC5) in *Escherichia coli* based on the proposed cleavage site that yields 12.5 kDa peptide to study its antigenicity and other biological functions *in vitro*. We purified recombinant human irisin (rh-irisin) to 95% homogeneity with simple purification method with a yield of 25 mg/g wet cell pellet. rh-irisin has been detected by commercially available antibodies from different sources with similar antigenicity. Biological activity of the rh-irisin was confirmed by using 3T3-L1 pre-adipocyte differentiation by Oil red O staining. Further, rh-irisin treatment on pre-adipocytes showed increased expression of markers associated with energy expenditure. As it is involved in energy expenditure process, it could be considered as potential therapeutic option for various metabolic diseases.

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1. Introduction

Irisin, a recently identified adipo-myokine [1], is cleaved proteolytically from Fibronectin type III domain containing 5 (FNDC5) into extracellular milieu with unknown mechanism. Increased levels of FNDC5 mRNA were reported after exercise [1]. Exercise induced beneficial effects were well documented such as decreased insulin resistance, improved functions of heart, brain, adipose tissue etc. The mouse FNDC5 consists of a signal peptide (29 amino acids (aa)), a single fibronectin type III domain (94 aa), a region of 28 aa of an unknown structure and function, a transmembrane domain (19 aa) and a cytoplasmic domain (39 aa) [2].

A putative cleavage site is proposed in the region of 'unknown structure and function' in FNDC5 [3]. After cleavage, the irisin travels through the blood and reaches the adipose tissue where it binds to an unknown receptor and facilitates the browning of white adipose tissue (WAT) [1].

While WAT is known for storage of energy, brown adipose tissue (BAT) is involved in thermogenesis. Moreover, BAT is characterized by more number of mitochondria and multilocular lipid droplets [4]. Recently, a third type of adipocytes has been identified in rodents and humans upon the thermogenic stimuli of WAT and are named as beige or brite (brown in white) adipocytes. However, the precursor cells of beige adipose cells are not similar to classical BAT but closer to WAT [5]. Above all, the thermogenic capacity is also much lower than the classical BAT [6]. However, the beige adipocytes showed increased number of mitochondria and UCP-1 mRNA levels which indicates increased energy expenditure.

Bostrom et al., suggested that the main function of irisin could be conversion of WAT into brown like adipose tissue (beige adipose

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tissue) [1]. After identification of irisin, many studies have been carried out to unravel its role in physiological functions.

Various sizes of irisin have been reported in the earlier studies. Bostrom et al., demonstrated the size of irisin as 32 kDa (glycosylated form) by using Abcam antibodies, developed against the cytoplasmic region (148–179 aa) of FNDC5 [1]. They also showed 25 kDa irisin band with the Phoenix antibody (42–112 aa). In another study irisin size has been reported as faint band of 12 kDa [7]. As different sizes of irisin have been used to study the physiological roles *in vitro* and *in vivo* [3], it is still not clear as which form of irisin is functional [8].

In this study, we cloned and expressed a 12.5 kDa human irisin (rh-irisin) in *Escherichia coli* (*E. coli*) and tested its biological activity in terms of antigenicity and markers associated with energy expenditure and browning of adipose tissue. Upon treatment with rh-irisin on pre-adipocytes, increased levels of UCP-1 and TMEM26 are observed, considered to be the markers of thermogenesis and presence of beige adipocytes respectively.

2. Methods

2.1. Cloning and expression of human irisin

A DNA fragment, synthesized based on the *E. coli* codon usage (NP_001165411.2), harboring the open reading frame of human irisin (32–143 aa of FNDC5) was amplified using specific primers containing *Nde* I and *Eco*R I restriction sites. The amplified product for full length irisin (355 bp) was cloned into pET21a(+) by using *Nde* I and *Eco*R I restriction enzymes (Fig. 1A). The recombinant plasmid was transferred into competent (*E. coli* DH5 α) cells and grown in Luria-Bertani (LB)-antibiotic (ampicillin) medium overnight and the cloned cells bearing the recombinant plasmid (transformed cells) were confirmed by restriction digestion analysis and

DNA sequencing. The pET21a(+)-irisin (Fig. 1B) was isolated and transferred into expression host (BL21 (DE3) cells) for further expression and purification studies.

2.2. Expression of recombinant irisin in *E. coli*

Expression of cloned fragment was driven by T7 promoter, which can be regulated by inducing with isopropyl β -D-thiogalactopyranoside (IPTG), thereby allowing high-level expression of the rh-irisin. Single colony was isolated from the transformed colonies of *E. coli* from the agar plate and inoculated into 15 ml LB medium with ampicillin (100 μ g/ml) in 100 ml flask. The flasks were incubated at 37 °C in an orbital shaker at 250 rpm until the optical density of 0.8 at 600 nm. One ml of culture is collected and labelled as uninduced sample (0 h). The rest of the culture was induced with 0.5 mM IPTG for 4 h and samples were collected at every one h interval and analysed by 15% SDS-PAGE.

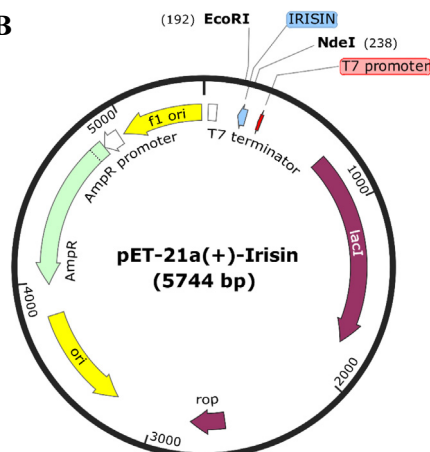
2.3. Purification of recombinant irisin

Purification of rh-irisin was carried out by the following procedure. The cells were induced with 0.5 mM IPTG and the culture (100 ml) was grown 4 h at 37 °C. The cells were harvested at 4000 rpm for 10 min at 4 °C, cell pellet (2 g) was collected and suspended with 4 ml of solubilisation buffer (50 mM Tris-Cl, 25% sucrose, 1 mM NaEDTA, pH: 8.0). The contents were mixed properly by vortexing and were centrifuged at 20,000 rpm for 20 min at 4 °C. Ice-cold sterile water (4 ml) was added to the pellet and cells were dispersed properly and cell pellet was collected at 20,000 rpm for 20 min at 4 °C and this step was repeated thrice. The pellet was suspended in 4 ml of lysis buffer (50 mM Tris-Cl, 1% Triton X-100, 1% sodium deoxycholate, 100 mM NaCl, pH: 8.0)

A

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CATATGGATAGCCCGTCTGCTCCGGTAAATGTCACGGTGCATCTGAAAGCGAACTCAGCCGTGGTTTCGTGGGATGT
GCTGGAAGACGAAGTCGTGATTGGCTTTGCAATCAGTCAGCAGAAAAAGACGTGCGTATGCTGCGCTTCATTCAGGAA
GTTAATACCACGACCCGTAGCTGCGCGCTGTGGGATCTGGAAGAAGACACGGAATATATCGTTTACGTCCAAGCGATTTC
TATCCAGGGTCAAAGTCCGGCCTCCGAACCGGTGCTGTTTAAACCCCGCGGAAGCAGAAAAATGGCTTCCAAAAA
CAAAGATGAAGTTACGATGAAAGAATGAGAATTC.
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B



C

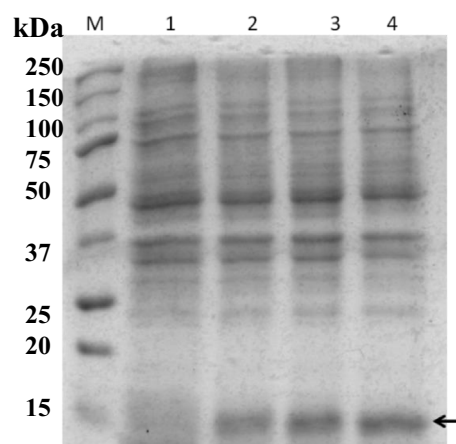


Fig. 1. A. The synthetic gene sequence of rh-irisin. B. Plasmid map of human *Irisin* gene cloned in pET21a(+) by *Nde* I and *Eco*R I. C. Expression analysis by SDS-PAGE of rh-irisin expressed in *E. coli*. Arrow indicates the expressed protein at 12.5 kDa. Lane M: protein marker, lane 1: before IPTG induction, show no protein expression at 12.5 kDa, lane 2: two hours after IPTG induction, lane 3: three hours after IPTG induction, lane 4: four hours after IPTG induction shows highest expression of rh-irisin.

and were allowed at room temperature for overnight and centrifuged at 20,000 rpm for 20 min at 4 °C. The pellet was washed with wash buffer with Triton X-100 (50 mM Tris-Cl, 0.5% Triton X-100, 1 mM NaEDTA, 100 mM NaCl, pH: 8.0) and centrifuged at 20,000 rpm for 20 min at 4 °C. The pellet was washed thrice with wash buffer without Triton X-100 (50 mM Tris-Cl, 1 mM NaEDTA, 100 mM NaCl, pH: 8.0), pellet containing inclusion bodies was collected at 20,000 rpm for 20 min at 4 °C and dissolved in 8 M urea. The dissolved proteins in urea were dialysed against PBS by placing the dialysis bag containing the protein in a beaker containing the PBS at slow stirring. The dialysed sample was refolded by adding drop by drop to the refolding buffer (100 mM Tris-Cl, 400 mM L-arginine, 2 mM NaEDTA, 0.5 mM oxidised glutathione, 5 mM reduced glutathione, 50 µl of protease inhibitors cocktail) with slow stirring at 4 °C for 8 h. The refolded protein was precipitated using 80% ammonium sulphate. After adding the required amount of ammonium sulphate, the solution was allowed at 4 °C with moderate stirring for 3–4 h. Then the precipitate was collected by centrifugation at 12,000 rpm at 4 °C for 20 min. The pellet was dissolved in 2 ml of sterile PBS and concentrated using the molecular weight cut-off membrane (3 kDa size). The purity of the rh-irisin was analysed by 15% SDS-PAGE.

2.4. rh-irisin purity analysis by analytical RP-HPLC

One hundred micro litres of purified rh-irisin (0.5 mg/ml) was loaded on analytical HPLC (LC-2010C HT[®] system, Shimadzu Corporation, Kyoto, Japan) connected to reversed phase C4 analytical column (150 × 4.6, 5 µ particle size, 30 nm pore size; Grace Vydac) using solvent A (0.1% TFA) and solvent B (90% acetonitrile with 0.1% trichloroacetic acid) with linear gradient of B composition (0 to 30 min: 36–55% of B, 30 to 35 min: 56–100% of B; 35 to 45 min: 100% of B and 45 to 50 min: 100–36%, 50 to 60 min 36%) with flow rate of 1.2 ml/min, samples were detected at 214 nm.

2.5. Cell proliferation assay

The effect of *E. coli* derived rh-irisin on 3T3-L1 cell proliferation was determined by measuring the activity of mitochondrial dehydrogenase-enzyme of living cells, where the enzyme converts the tetrazolium bromide (MTT) into a purple formazan product and the intensity of the colored product was measured by using spectrophotometry as described previously [9]. Briefly, 3T3-L1 cells (3×10^5 cells/ml) were cultured in DMEM medium containing 10% FBS, 100 U penicillin/ml, 100 µg/ml streptomycin (Gibco) and incubated at 37 °C for 24 h with 5% CO₂ in 96 well culture plate. Cells were treated with or without purified rh-irisin for 72 h. The dose of irisin was selected based on the previous literature [10]. After each 24 h time point 25 µl of 0.5% sterile tetrazolium bromide was added to each well and re-incubated for 3 h. Then, 100 µl of a 24% sodium dodecyl sulfate was added to each well and again re-incubated for 30 min. Absorbance was read at 590 nm using microplate reader (Bio-Rad).

2.6. 3T3-L1 differentiation and Oil red O staining

3T3-L1 pre-adipocytes were grown and maintained in DMEM containing 10% FBS. 3T3-L1 cells were plated at 10^5 cells per well in a six well plate and allowed to become confluent for 48 h. After cells become confluent, cells were treated with medium containing rosiglitazone (Rosi, 5 µM) or rh-irisin (20 nM and 50 nM) for 48 h. After treatment, cells were fed with medium containing Rosi or rh-irisin for another 48 h and subsequently with plain medium for 48 h as described previously [11]. These cells were then stained with

Oil red O and images of the cells on the plate were taken using inverted phase-contrast microscope.

2.7. Western blotting

The protein was collected from the 3T3-L1 cells after treatment with rh-irisin. After washing with cold PBS, cells were lysed in Tris-Cl pH 8.0, Triton X-100 0.1% and 150 mM NaCl containing protease inhibitors. Total protein was obtained after 3 cycles of freeze-thaw and centrifugation at 12,000 rpm for 15 min at 4 °C and stored at –80 °C until further use. Total protein content was estimated by BCA protein assay kit (Pierce, Rockford, IL). Equal amounts (20 µg) of protein and transferred to PVDF membrane. For antigenicity testing, purified rh-irisin (0.1 to 7.5 µg) was used. The membrane was blocked with 5% BSA and then probed with anti-irisin or anti-UCP-1 antibodies overnight at 4 °C. Then the membrane was washed with TBS-T buffer and probed secondary antibody conjugated with HRP for 1 h at room temperature. The proteins were visualized using Pierce ECL Western blotting substrate (Thermo Fisher Scientific, PA) and photographs were captured with x-ray films.

2.8. Antigenicity testing of irisin by ELISA

To test the antigenicity of the purified rh-irisin, ELISA was performed. Purified protein in PBS (10, 20 and 50 ng in 100 µl) was coated in a flat bottom 96 well plate (Nunc MaxiSorp, Thermo Fisher Scientific) and incubated overnight at 4 °C. Commercial human irisin (50 ng, Cayman Chemicals) was used as a positive control. The plate was washed with wash buffer (1 X PBS with 0.05% Tween 20) for three times followed by blocking with 1% BSA for 1 h at room temperature. Then anti-irisin antibody (Cayman chemicals, 1: 1000) was added and incubated for 2 h at room temperature followed by secondary antibody conjugated with HRP for 1 h at room temperature. After each incubation, plate was washed three times with wash buffer. Finally, the plate was developed with 100 µl of TMB (3,3',5,5'-tetramethylbenzidine) substrate buffer, and reaction was stopped after 30 min by adding 50 µl of 4 N H₂SO₄. Absorbance was read at 450 nm using microplate reader (Bio-Rad).

2.9. RNA isolation and qRT-PCR

Total RNA was isolated from 3T3-L1 cells after treatment with or without rh-irisin or rosiglitazone at indicated time point using TRIzol reagent and purified by Qiagen purification kit as per manufacturer's instruction. RNA was quantified by Nanodrop2000 and purity was checked by 260/280 ratio. One microgram of total RNA was used for cDNA synthesis. cDNA synthesis was performed by reverse transcriptase (RT) enzyme obtained from Takara biosciences. cDNA was diluted to 1:5 and 5 µl was used for semi quantitative or quantitative RT-PCR (qRT-PCR) using Sybr green kit obtained from the Bio-Rad. Relative fold change in UCP-1, PGC-1 α and TMEM26 transcripts was calculated using ddCT method [12]. PGC-1 α and betatrophin (ANGPTL8) was measured by semi-quantitative RT-PCR. PCR product was separated on 1.5% agarose gel electrophoresis and stained with ethidium bromide. Captured image density was analysed by NIH-ImageJ software. The primers used for RT-PCR and qRT-PCR were listed in Table 1.

2.10. Statistical analysis

The data were presented as mean \pm SEM of at least three independent experiments and were analyzed using Student's *t*-test by using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA). *P* < 0.05 was considered statistically significant.

Table 1
Primer sequences for the qRT-PCR.

	Gene	Forward primer 5' → 3'	Reverse Primer 5' → 3'
1	UCP1	CAAAAACAGAAGGATTGCCGAAA	TCTTGACTGAGTCGTAGAGG
2	PGC-1 α	CCCTGCCATTGTTAAGACC	TGCTGCTGTTCTGTTTTTC
3	Betatrophin	CAGGATGCCACACAGGAGCTTC	TCAGCTGCACTGTAGTCTC
4	Beta-actin	CGCCACCAGTTCGCCATGGA	TACAGCCCGGGGAGCATCGT
5	TMEM26	TTCTGTTCATTCCTGGTC	GCCGGAGAAAGCCATTGT

3. Results

3.1. Expression analysis of human recombinant irisin

In order to produce authentic human irisin in *E. coli* expression system, we designed and constructed an optimized DNA sequence for *E. coli* codon usage. The synthesized DNA sequence of rh-irisin is shown in Fig. 1A. The synthetic DNA was amplified with specific primers containing *Nde* I and *Eco*R I restriction sites, digested with *Nde* I and *Eco*R I followed by ligation with pre-digested pET21a(+) to construct a recombinant pET21a(+)-irisin- (Fig. 1B). After transforming the ligated products into competent *E. coli* cells, the antibiotic resistant colonies were screened by restriction digestion and confirmed by DNA sequencing (data not shown). To examine the irisin expression, the *E. coli* cells were cultured in LB medium and protein expression was induced by IPTG. Cells were collected at different time points and analysed the cell lysate by SDS-PAGE. The rh-irisin was expressed highest at 4 h at ~12.5 kDa after IPTG induction and no expression was observed in un-induced samples (Fig. 1C). Densitometry analysis of the total lane (Fig. 1C, lane 4) showed rh-irisin expressed in *E. coli* demonstrated to be ~28% of the total cell proteins (168 mg/2 g wet cell mass). The size of rh-irisin was correlated with calculated size from deduced amino acid sequence and found to be consistent with previous reports [7]. The solubility of the rh-irisin expression was analysed and expressed as inclusion bodies (data not shown).

3.2. Purification human recombinant irisin

Cell pellet (2 g) was washed and lysed to prepare insoluble aggregates, inclusion bodies. Inclusion bodies (150 mg) were thoroughly washed to remove host proteins, DNA and endotoxins [13]. The inclusion bodies were solubilised with 8 M urea and dialysed against PBS with a step yield of 90 mg. After refolding and concentration, the yield was found to be 50 mg/2 g of wet cell mass (Table 2). The final purified protein was analysed on 15% SDS-PAGE, which showed as single band at 12.5 kDa (Fig. 2A; lane 4). Further analysis of the gel by densitometry showed the band purity around 95%. Then we went on to check the purity by analytical reversed phase HPLC using butyl silyl (C_4) silica gel. The chromatogram shows three peaks of which the main peak corresponds to rh-irisin (Fig. 2B). The purity was observed as ~95% and each

individual impurity was identified less than 5% (Fig. 2B and Peak table).

3.3. Antigenicity testing of purified rh-irisin

The purified rh-irisin was biologically assessed to know whether it has antigenicity and study interactions with commercially available antibodies to irisin. The antigenicity of the rh-irisin was assessed by Western blotting as well as ELISA. Different concentrations (0.1, 2.0, 5.0 and 7.5 μ g /lane) of purified rh-irisin was loaded and probed with anti-irisin antibody (Cayman chemicals). Increased band intensities were observed on the blot at the expected size of 12.5 kDa (Fig. 3A). These results indicate that the purified rh-irisin has good antigenicity. To further validate the antigenicity, we used rh-irisin from three different batches and commercially available antibodies from three different sources (A. Cayman chemicals; B. Abcam; and C. Lifespan Biosciences Inc) which were raised against different regions/epitopes of FNDC5. It was found that the antibodies detected the purified rh-irisin in similar passion, size and highlighting the antigenicity of purified rh-irisin (Fig. 3B). Further, its antigenicity was confirmed by ELISA and it is comparable with commercial irisin (Fig. 3C). This data conclusively demonstrate that the purified rh-irisin (12.5 kDa) has antigenicity.

3.4. Effect of recombinant irisin on 3T3-L1 cell proliferation

In order to check the effect of rh-irisin on cell proliferation, 3T3-L1 cells were cultured in 96 well plate and treated with different concentrations (0, 20, 50 and 100 nM) of irisin and continued for 72 h. Proliferation was analysed by adding MTT reagent and the absorbance was measured at 590 nm. The data revealed that the proliferation ability among the treated groups showed no significant difference indicating no toxicity (Fig. 4A).

3.5. Recombinant irisin stimulates the differentiation of 3T3-L1 derived adipocytes

Irisin has been reported to have role in energy expenditure and induced the browning of WAT. Further to its antigenicity, the biological significance of the purified rh-irisin was tested by using *in vitro* adipogenesis model system with 3T3-L1 cell line. The

Table 2
Yields and purities of recombinant human irisin (rh-irisin) achieved at each step of purification from 2 g of wet cells (100 ml of *E. coli* culture) (data shown is an average of three different batches).

Step	Purification step	Total protein (mg)	rh-irisin (mg) (A)	Purity (%)	Yield (%) (C)
1	Whole cell	600	168	28(B)	100
2	Purified IBs after Cell lysis	150	120	80(B)	71
3	IB solubilization	115	97	85(B)	80
4	Refolding and Concentration	90	50	95(D)	51

Total protein includes both soluble and insoluble protein.

A. Total protein includes both soluble and insoluble protein.

B. Purity was determined by densitometry from SDS-PAGE gels.

C. Yield was calculated considering irisin protein quantity as a 100% from step 1.

D. Purity of rh-irisin was determined by analytical RP-HPLC.

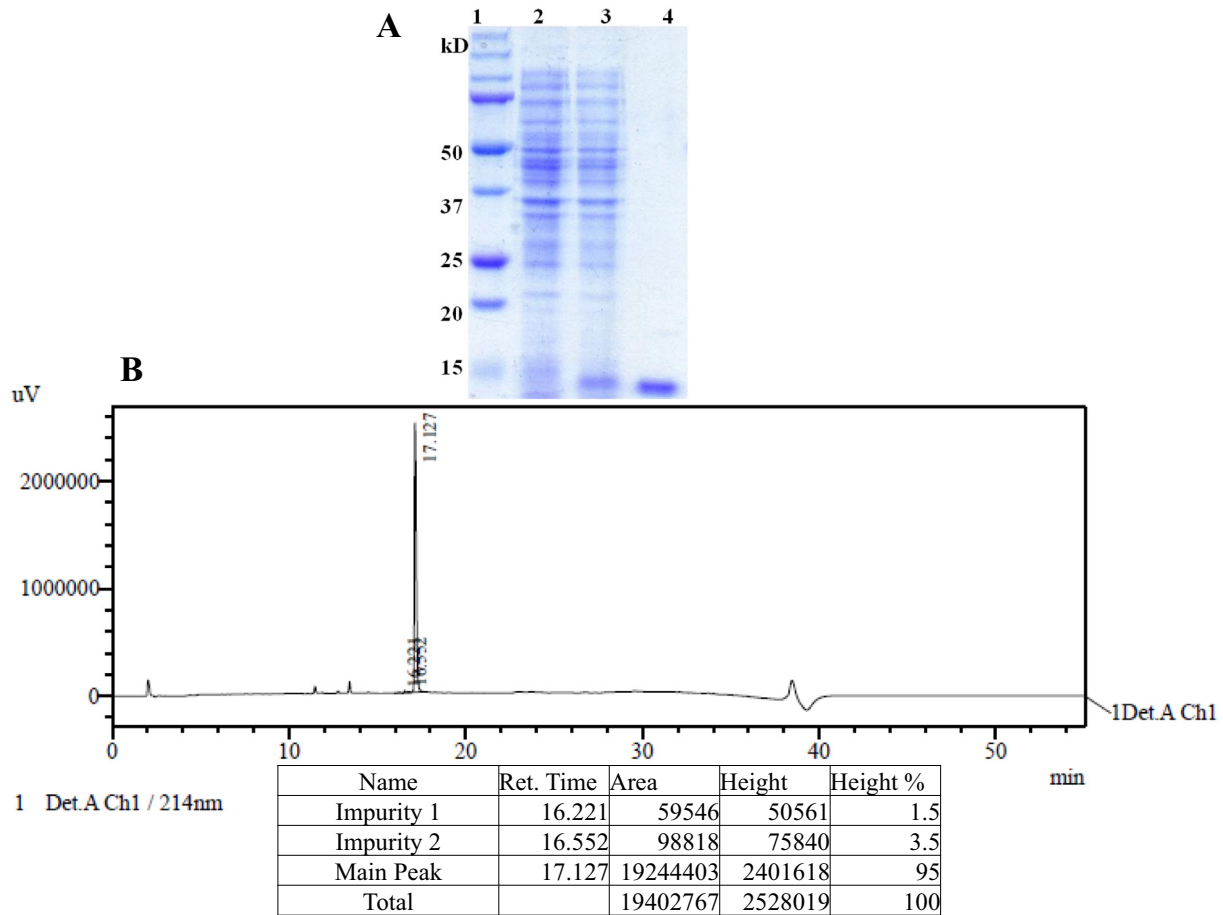


Fig. 2. **A:** SDS-PAGE analysis of expressed and purified samples. Lane 1: protein marker, lane 2: before IPTG induction, shows no protein expression at 12.5 kDa, lane 3: four hours after IPTG induction, lane 4: after refolding and concentration, the final purified recombinant human irisin (rh-irisin) shows single band at 12.5 kDa. **B:** Representative chromatogram of reversed phase chromatography (RPC) analysis of purified rh-irisin. Final protein was loaded on the RPC column and percentage of each peak was analysed. Peak 3 represent the rh-irisin and show ~95% pure and the remaining minor peaks are impurities represent less than 5%. Representative peak purity table shown below.

confluent 3T3-L1 cells were treated with different concentrations of the rh-irisin and after 10 days, the cells were fixed for Oil red O staining. As shown in the Fig. 4B, rh-irisin induced the adipocyte differentiation at dose dependently as evidenced by Oil red O staining (Fig. 4B: B, C). Control cells treated with vehicle did not show any differentiation as evidenced no Oil red O stained cells (Fig. 4B: A). Rosiglitazone, known ligand for PPAR γ to induce the adipogenesis, was used as positive control (Fig. 4B: D).

3.6. Recombinant irisin regulates the expression of markers associated with browning of adipocytes

To investigate the browning effects of rh-irisin on adipocytes, we used 3T3-L1-derived adipocytes as cellular model. These are treated without or with rh-irisin as described in the methods. PGC-1 α is a powerful transcriptional co-activator, which is a master regulator of mitochondrial biogenesis, inducing UCP-1 and FNDC5 in brown fat cells. PGC-1 α mRNA expression was assessed by semiquantitative RT-PCR and qRT-PCR. rh-irisin treatment led to rapid upregulation of PGC-1 α mRNA expression on par with rosiglitazone treatment (Fig. 5A). Similar trend was observed with qRT-PCR analysis (Fig. 5B). Transmembrane protein 26 (TMEM26) is a cell surface marker for beige adipocytes [5,14]. TMEM26 mRNA expression was assessed by qRT-PCR. rh-irisin treated pre-adipocytes showed increased expression of TMEM26 compared to rosiglitazone treated cells, which indicates the conversion of white adipocytes into beige adipocytes (Fig. 5C).

Similarly, we analysed the UCP-1 expression at mRNA and protein level. UCP-1 is a mitochondrial protein that dissipates chemical energy to heat. There is significant dose dependent upregulation of UCP-1 was observed with irisin treatment by Western blotting (Fig. 6A) and two-fold increase in UCP-1 mRNA expression by qRT-PCR analysis (Fig. 6B). As reported earlier, irisin treatment upregulates the betatrophin (Angptl-8) [10], a newly identified molecule involved in regulation of plasma triglyceride levels and lipid metabolism [15]. We also measured the levels of betatrophin mRNA and found increased expression of betatrophin (Fig. 6C) and further, densitometry analysis revealed the difference in expression was significant compared to control (Fig. 6D). Interestingly, no change was observed in expression with rosiglitazone compared to control (Fig. 6D). These findings indicate that purified human irisin expressed in *E. coli* shows clear upregulation of markers associated with energy expenditure [10].

4. Discussion

We demonstrated that authentic human irisin synthetic DNA fragment was designed, cloned for high expression and yield. Simple purification system was reported for the evaluation of its biological activity in terms of energy expenditure markers. Recent reports indicate that irisin promotes brown fat development and thermogenesis in white adipocytes both *in vitro* and *in vivo* [8]. The discovery of this adipokine has given scope for understanding

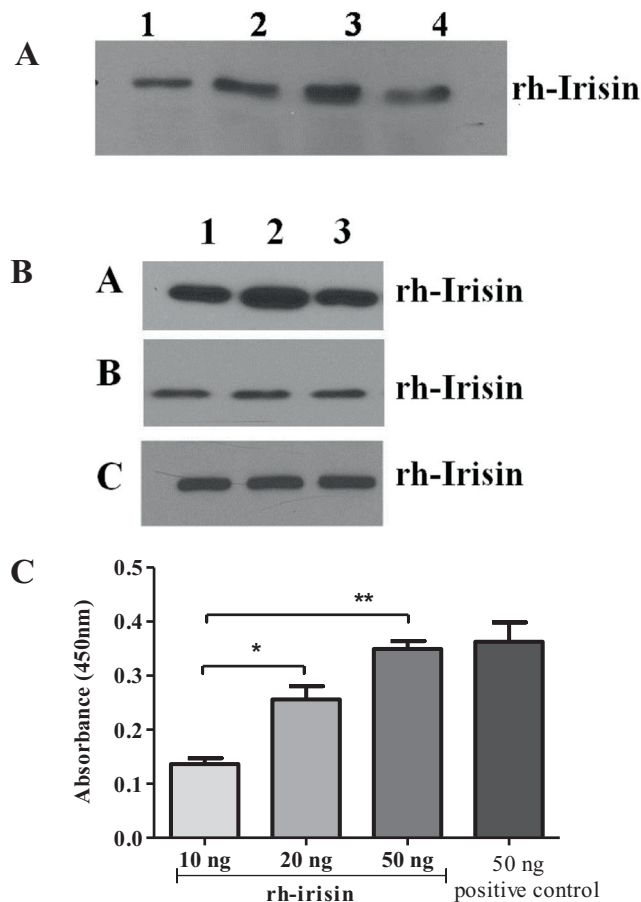


Fig. 3. Western blot analysis of purified recombinant human irisin (rh-irisin) at different concentrations and different source of antibodies. **A.** Pure rh-irisin (lane 1: 0.1, lane 2: 2.0, lane 3: 5.0 and lane 4: 7.5 μ g) was separated on 15% SDS-PAGE and transferred to nitrocellulose membrane and probed with anti-irisin antibodies (Cayman chemicals). **B.** Five micrograms of purified irisin from different purification batch was separated on 15% SDS-PAGE and transferred to nitrocellulose membrane and probed with anti-irisin antibodies (A. Cayman chemicals; B. Abcam; and C. Lifespan Biosciences Inc.). **C.** Antigenicity of purified rh-irisin was confirmed by ELISA. Three different concentrations of rh-irisin (10, 20 and 50 ng) and 50 ng of known irisin (Positive control; Cayman chemicals) were used for this assay. Data is reported as mean \pm SEM. *p-value < 0.05 vs. 10 ng, **p-value < 0.01 vs. 10 ng.

its role in metabolic conditions such as diabetes, obesity etc. In this study, the purified rh-irisin shows 95% purity and greater antigenicity against commercial antibodies. Further, biological activity showed that rh-irisin differentiates 3T3-L1 adipocytes and induces the expression of markers related to beige adipocytes and energy expenditure.

The theoretical molecular weight of irisin was found to be 12.6 kDa [1]. However, different studies have reported varied sizes of irisin (22 kDa, 25 kDa) by Western blotting using commercial antibodies to irisin/FNDC5. Lee et al., have used mass spectrometry to identify irisin in human serum samples and revealed glycosylated and deglycosylated forms of FNDC5/irisin at 32 kDa and 24 kDa respectively [25]. However, in an earlier study, different molecular weights of irisin have been reported with site directed mutation (16 kDa), irisin dimer (23.5 kDa) and glycosylated irisin (36 kDa) by size exclusion chromatography [16]. In an attempt to answer the contradictions about size of irisin, we have designed human irisin gene according to the sequence as reported earlier [1], and purified to study its biological role. In this study, we showed the expression of irisin at 28% of total proteins, which is comparatively high in *E. coli* expression systems [17]. In another study, irisin was produced with a GST tag in *E. coli* and analysed the biological activity of GST-irisin on adipogenesis [18]. But, they did not report the expression level of irisin, besides using tagged-irisin for biological assessment as tag may sometimes interfere with biological functions. In this study, we expressed the irisin (12.5 kDa) without any tags as inclusion bodies. These inclusion bodies were efficiently solubilised and purified with a simple purification method, with removal of host proteins and DNA contaminants [13]. The endotoxin levels in the final purified protein were reported to be less than 5 U/ml as assessed by the Limulus ameocyte lysate test [18,19]. The purified rh-irisin analysed by RP-HPLC was found to be 95% pure, with contaminants accounted for less than 5% in the final purified protein.

Then, we assessed the biological roles of purified rh-irisin on various parameters associated with browning of the adipocytes. In order to ascertain its cytotoxicity, we exposed 3T3-L1 cells to different concentrations of purified irisin for 3 days. Our MTT data revealed that purified irisin has no significant effect on cell proliferation suggesting negative cytotoxic effects. Moreover, rh-irisin has efficiently differentiated 3T3-L1 pre-adipocytes as assessed by Oil red O staining testifying its full-biological function [10].

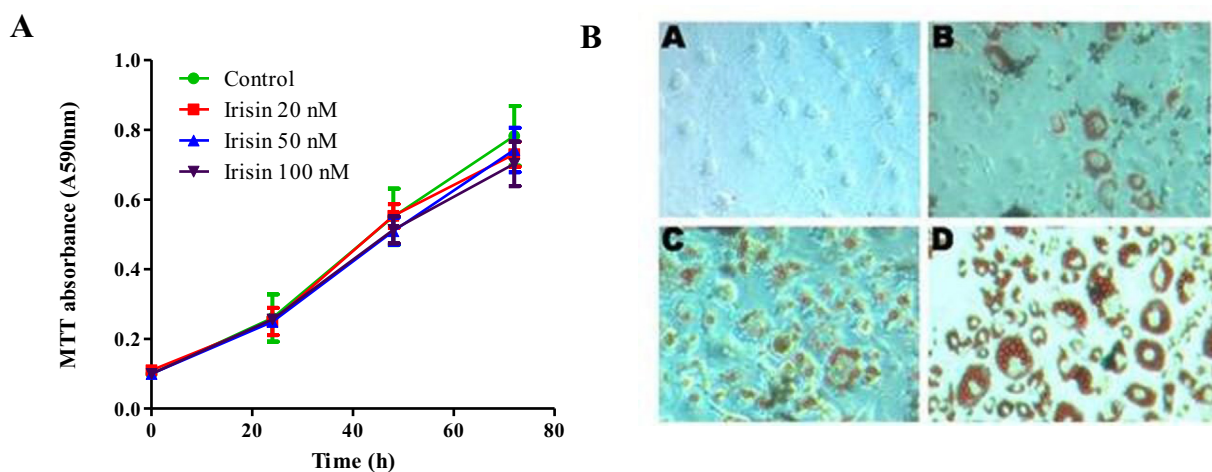


Fig. 4. **A.** 3T3-L1 cells proliferation assay by MTT assay following recombinant human irisin (irisin) treatment. Cells were cultured and treated with three different concentrations (20, 50 and 100 nM) for 3 days and every 24 h cell proliferation was measured and the absorbance was plotted. Data is reported as mean \pm SEM. **B.** Recombinant human irisin (irisin) activates the differentiation of 3T3-L1 cells into adipocytes as evidenced by the Oil red O staining on day 10 of differentiation. **A.** Control cells without any differentiation media; **B.** Irisin at 20 nM; **C.** Irisin at 50 nM; **D.** Rosiglitazone (Rosi, 5 μ M) as a positive control. Data is a representative of three independent experiments.

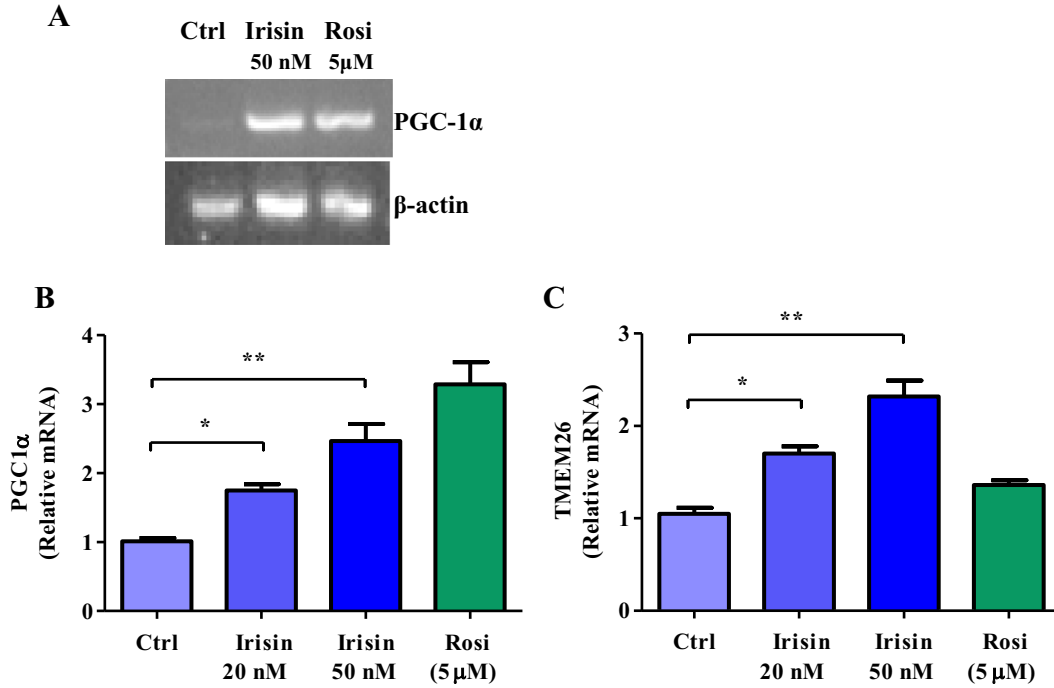


Fig. 5. Recombinant human irisin (irisin) expressed in *E. coli* regulates the expression of adipocyte-specific PGC-1 α mRNA, TMEM26 in 3T3-L1 cells. **A.** Total RNA was extracted from the cells and cDNA was synthesized as described in the methods. RT-PCR was carried out using specific primers for PGC-1 α and product was analysed on agarose gel electrophoresis. Beta actin was used as reference gene. Rosiglitazone (Rosi, 5 μ M) treated cells were used as a positive control. **B.** In another set of experiments, cDNA was used for quantitative RT-PCR of PGC-1 α as described in the methods. **C.** In another experiment, cDNA was used for quantitative RT-PCR of TMEM26. Data is reported as mean \pm SEM. *p-value < 0.05 vs. control (Ctrl), **p-value < 0.01 vs. control. Data is a representative of three independent experiments.

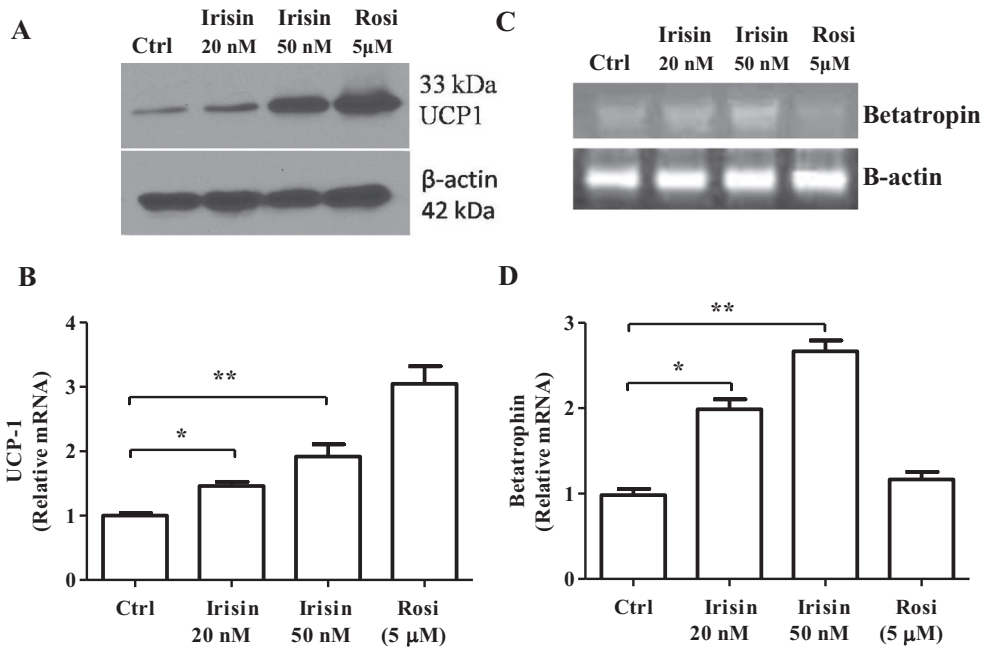


Fig. 6. Recombinant human irisin (irisin) regulates the expression of brown adipocyte-specific UCP-1 and betatropin in 3T3-L1 cells. UCP-1 expression was measured by **A.** Western blot and **B.** quantitative RT-PCR as described in the methods. Betatropin expression was analysed by RT-PCR (**C.**) and densitometry analysis was carried out to quantify the expression by ImageJ software (**D.**). Rosiglitazone (Rosi, 5 μ M) treated cells were used as a positive control. Data is reported as mean \pm SEM. *p-value < 0.05 vs. control (Ctrl), **p-value < 0.01 vs. control. Data is a representative of three independent experiments.

There are two distinct types of brown adipose cells, first one is the classical brown fat that arises from a muscle-like cell lineage (*myf5*) and other one is the brown adipocytes that found interspersed within the WAT [20–22]. Brown cells present in WAT, which express UCP-1 are designated as beige or brown-in-white

(brite) cells [23]. In the present study, we demonstrated that rh-irisin treatment indeed increased the expression of thermogenic genes including PGC-1 α and UCP-1 in 3T3-L1 derived adipocytes. The expression of UCP-1 is regulated by several transcription factors including PGC-1 α , which can be induced by cold exposure

and/or β -adrenergic signalling [24]. The results underlines the active participation of purified rh-irisin in induction of thermogenic brown adipocyte markers UCP-1 and PGC-1 α corroborative with the previous studies [10]. Further to understand whether rh-irisin could induce the beige cell marker, TMEM26, we analysed the rh-irisin treated cells for mRNA of TMEM26 by qRT-PCR. Our results showed that TMEM26 levels were increased when rh-irisin was added to the cultured 3T3-L1 pre-adipocytes during differentiation. Previous studies also established that preferential sensitivity of beige cell precursors to the browning effect of irisin [5]. Influence of rh-irisin in the formation of beige cells is confirmed and consistent with previous reports performed with rh-irisin expressed in *Pichia pastoris* [10].

5. Conclusions

In the present study, we cloned, expressed and purified human irisin (12.5 kDa) to study its antigenicity and biological role in energy expenditure. Irisin treatment induced the expression of thermogenic genes such as PGC-1 α , UCP-1 and TMEM26 during differentiation of the 3T3-L1 pre-adipocytes. Further, it has highlighted the 12.5 kDa irisin role in energy expenditure. Antibodies raised to this region could also be used to detect the irisin levels in various biological samples to study its physiological role.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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