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Transgenic expression and recovery of biologically active recombinant human insulin from *Arabidopsis thaliana* seeds

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Summary

The increased incidence of diabetes, coupled with the introduction of alternative delivery methods that rely on higher doses, is expected to result in a substantial escalation in the demand for affordable insulin in the future. Limitations in the capacity and economics of production will make it difficult for current manufacturing technologies to meet this demand. We have developed a novel expression and recovery technology for the economical manufacture of biopharmaceuticals from oilseeds. Using this technology, recombinant human precursor insulin was expressed in transgenic plants. Plant-derived insulin accumulates to significant levels in transgenic seed (0.13% total seed protein) and can be enzymatically treated *in vitro* to generate a product with a mass identical to that of the predicted product, $DesB_{30}$-insulin. The biological activity of this product *in vivo* and *in vitro* was demonstrated using an insulin tolerance test in mice and phosphorylation assay performed in a mammalian cell culture system, respectively.

Introduction

Approximately 0.7% of the world's population suffers from insulin-dependent diabetes mellitus (IDDM; diabetes mellitus Type I) (Winter *et al*., 2001). It has been estimated that the incidence of diabetes will double to approximately 300 million in the next 25 years (Kjeldsen *et al*., 2001). Currently, diabetes mellitus is the third largest cause of death in industrialized countries, after cardiovascular diseases and cancer (Barfoed, 1987). To date, insulin therapy is the only effective treatment for Type 1 diabetes and is also generally required for the treatment of Type 2 diabetes as the disease progresses. Therapy requires the regular monitoring of blood glucose levels, combined with frequent injection of insulin, in order to avoid the severe debilitating secondary complications associated with chronic hyperglycaemia. Unfortunately, the discomfort and inconvenience of this rigorous self-injection regimen can lead to non-compliance. To overcome this problem, a variety of more patient-friendly alternative delivery methods are currently being developed, including the administration of insulin through pulmonary, nasal and oral routes (Modi *et al*., 2002; Goldberg and Gomez-Orellana, 2003; Cefalu, 2004). Although these non-invasive methods may increase compliance, they typically require 5–20-fold higher doses to compensate for reduced bioavailability. When combined with a rise in both the incidence and diagnosis of diabetes worldwide, the introduction of alternative delivery methods is anticipated to dramatically increase the demand for insulin (currently growing at 3%–4% per year). Meeting this demand will necessitate the development of more cost-effective, higher capacity production in the near future.

Recombinant human insulin has been expressed in a variety of different organisms, including bacteria (Chan *et al*., 1981), yeast (Thim *et al*., 1986), fungi (Wang *et al*., 2001), mammalian cell cultures (Yanagita *et al*., 1992) and transgenic plants (Arakawa *et al*., 1998). However, commercial production is currently limited to *Escherichia coli* (Chan *et al*., 1981) and *Saccharomyces cerevisiae* (Thim *et al*., 1986). Although these commercial systems have undergone considerable refinement and optimization over the past two decades, and have

achieved annual production levels on the order of five tons, they will be severely challenged to meet the capacity and economic demands of the future. Plant-based systems offer the potential for safe, economical, high-capacity production for many biopharmaceuticals (Deckers *et al*., 1999). However, given the attractiveness of insulin as a target, the limited number of citations in the literature suggests that previous attempts at its production in plants have met with little success. We have developed a unique plant-based production technology involving targeting to subcellular organelles known as oilbodies that enables both high levels of expression and cost-effective recovery of recombinant insulin.

Oilbodies are discrete storage organelles found in oilseeds, comprising a hydrophobic triacylglycerol core surrounded by a half-unit phospholipid membrane and an outer shell of specialized proteins known as oleosins. Our technology allows for the production of genetically engineered oil seeds, in which proteins are covalently targeted to oilbodies as oleosin fusions (Van Rooijen and Moloney, 1995; Deckers *et al*., 1999). This feature allows the separation of oilbodies from other seed components by liquid–liquid phase separation. This mild process eliminates the need for refolding and can reduce the number of chromatography steps required to obtain a purified product, thereby significantly reducing the overall cost of goods. As a proof-of-concept for this technology, we have produced recombinant human insulin in the model plant species *Arabidopsis thaliana*. Recombinant insulin from transgenic *Arabidopsis* seeds was matured *in vitro* using trypsin and further characterized by mass spectral analysis. The biological activity of the purified product was also assessed. Using our technology, we have demonstrated that insulin can be expressed and recovered as an active molecule at commercially relevant levels from transgenic seeds.

Results and discussion

Recombinant expression of insulin in transgenic seed

A. thaliana plants were transformed with the plant binary expression vector pSBS4405 (Figure 1). The construct encodes a fusion protein, oleosin–human insulin (OB-hIN), comprising recombinant human mini-insulin with an N-terminal trypsin-cleavable propeptide (Kjeldsen *et al*., 2001) (Klip27-mini-insulin) fused to the C-terminus of the *A. thaliana* 18-kDa oleosin-Klip8 polypeptide. We have previously shown, with a variety of different fusion partners, that such oleosin fusion proteins retain their native targeting to oilbodies *in vivo* and fractionate with oilbodies following aqueous extraction and separation (Van Rooijen and Moloney, 1995). The analysis of insulin expression was conducted on mature seeds harvested from transgenic *Arabidopsis* plants. Total extractable seed protein and purified oilbody protein, obtained using flotation centrifugation, were analysed to determine the expression levels of the OB-hIN fusion protein. Of 23 independent lines analysed, 15 were positive on the basis of Western blot analysis (data not shown). Figure 2 shows the expression of OB-hIN in the three highest transformed lines. A polypeptide corresponding to the 34.2-kDa molecular mass predicted for the fusion protein was clearly visible in the total protein profiles from transgenic seed (Figure 2a) and was highly enriched following oilbody purification (Figure 2b). The identity of the fusion protein was established using antibodies directed against insulin (data not shown). Differences in expression levels are the result of clonal variation amongst transformants. The expression levels were determined by densitometry using the native 18-kDa oleosin band as an internal standard (equivalent to 1.5% total seed protein). The expression of insulin in transformed seed was approximately six times higher than previously reported in plants (Table 1). In an earlier report, recombinant human insulin was synthesized as a fusion with the *cholera* toxin B (CTB) protein and expressed in potato tubers (Arakawa *et al*., 1998). The CTB–insulin fusion accounted for 0.1% of the total soluble protein in the transformed tissue, and therefore mature insulin corresponded to 0.022% of the total soluble protein present in the tubers. It should be noted, however, that this work was intent on developing a product to induce immunotolerance and thereby prevent the onset of diabetes, and therefore high levels of production were not required.

Plant-derived $DesB_{30}$-insulin was authenticated following maturation *in vitro* with trypsin

Current methods for the commercial manufacture of insulin in both *E. coli* and *S. cerevisiae* involve the enzymatic maturation from a precursor form of the protein through trypsin digestion (or similar enzyme) *in vitro* (Kjeldsen *et al*., 2001). To demonstrate that a similar process could be used for plant-based production, recombinant insulin was simultaneously cleaved from the oleosin fusion partner and matured *in vitro* with trypsin following oilbody purification. When analysed by reverse phase high-performance liquid chromatography (RP-HPLC), the products from this reaction included a peak with a retention time almost identical to that of the insulin standard (Table 2). Wild-type non-recombinant oilbodies prepared in the same manner did not resolve any elution peaks between 17.0 and 18.0 min (data not shown). The slight increase observed in the retention time was attributed

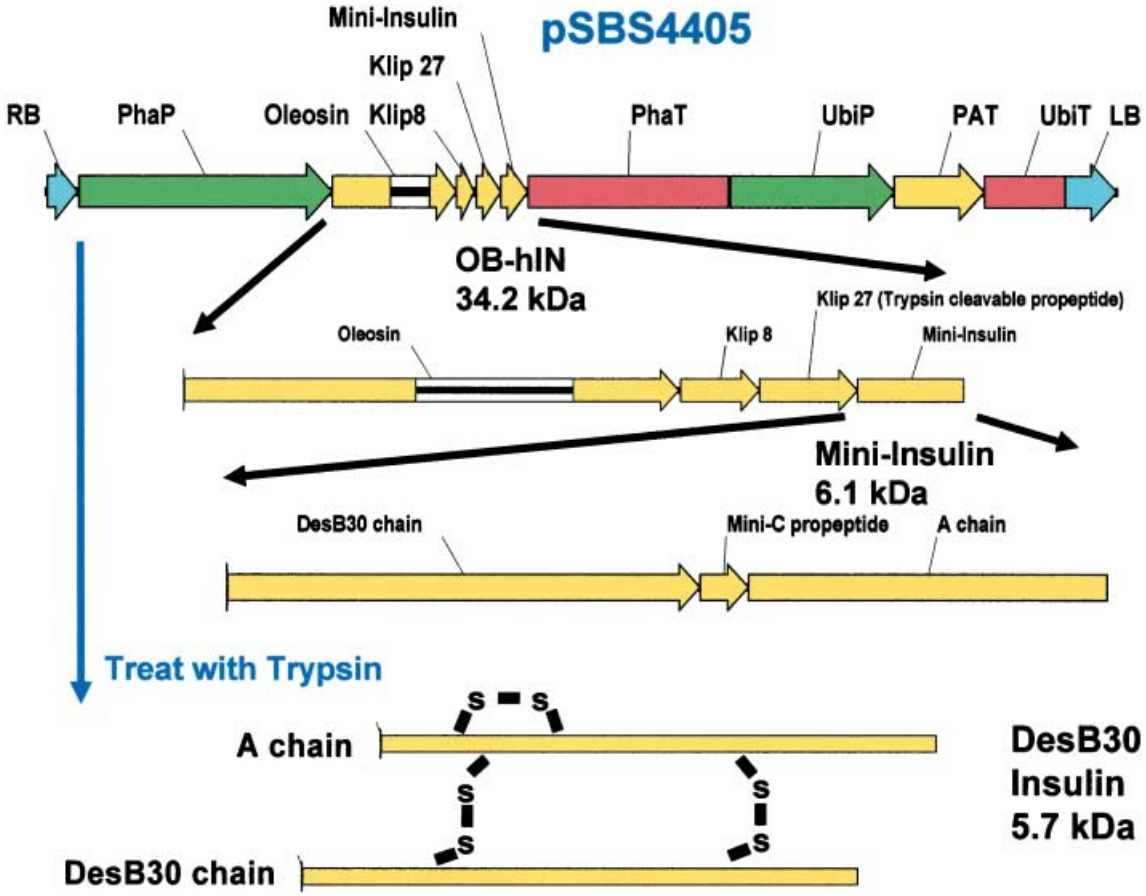
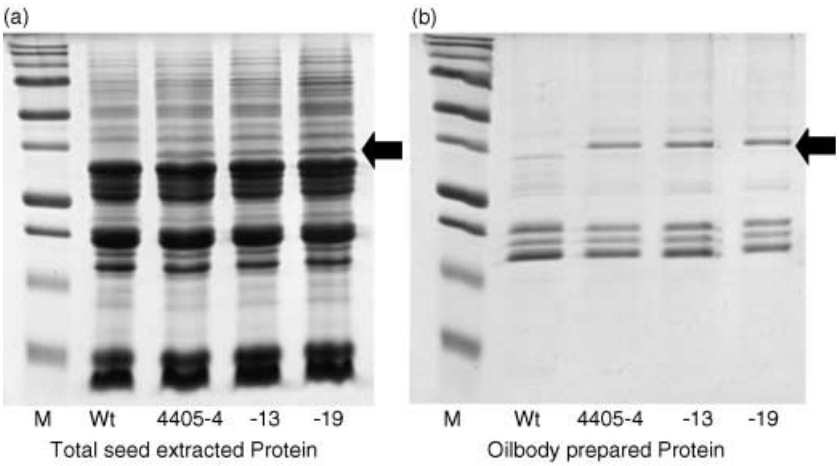


Figure 1 Plant expression vector pSBS4405 encoding for oleosin–human insulin (OB-hIN). The 34.2-kDa OB-hIN is encoded by the 18-KDa *Arabidopsis* oleosin genomic clone in frame with Klip8 followed by Klip27 (trypsin-cleavable propeptide) and mini-insulin. The mini-insulin is encoded by the $DesB_{30}$ chain and A chain of human insulin interposed by a mini-C propeptide (AAK) using plant codon usage. When the OB-hIN is treated with trypsin *in vitro*, the fusion protein is converted to $DesB_{30}$-insulin (5.7 kDa). RB, right border; PhaP, phaseolin promoter; PhaT, phaseolin terminator; UbiP, ubiquitin promoter; PAT, phosphinothricin acetyltransferase gene; UbiT, ubiquitin terminator; LB, left border.

Figure 2 Expression of oleosin–human insulin (OB-hIN) in transgenic *Arabidopsis thaliana* T_3 seeds. Expression of OB-hIN fusion proteins in three transgenic *A. thaliana* lines (OB-hIN; 4405-4, -13, -19) from total extractable seed protein (a) and oilbody purified samples (b) in Coomassie-stained sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Arrows denote the migrating fusion proteins. The identity of OB-hIN was established using Western blots probed with anti-insulin monoclonal antibody E2E3 (ab9569; Abcam, Cambridge, MA, USA) (data not shown). Wt refers to non-recombinant seed. The molecular weight markers (M) are 10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa from bottom to top, respectively.



to the difference in sequence between our recombinant human insulin protein product ($DesB_{30}$-insulin) and the full-length insulin standard. This conclusion was subsequently supported by results from mass spectrometry. The fraction collected from this peak was found to contain an immuno-reactive product of the same approximate molecular mass as the human insulin standard (data not shown). This fraction was further analysed by post-source decay matrix-assisted

Table 1 Oleosin human recombinant insulin transgene (OB-hIN) expression levels in T_3-4405 *Arabidopsis thaliana* seed determined by densitometry of Coomassie-stained sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The levels are expressed as the percentage of transgene and percentage molar hIN expression on a total seed protein basis. The percentage molar ratio of insulin to the fusion protein (0.17) was used to calculate the level of expression of hIN in transformed seed

Construct	Line	Fusion protein (OB-hIN) expression (% total seed protein)	hIN expression (% total seed protein)
pSBS4405	4	0.63	0.11
	13	0.62	0.11
	19	0.75	0.13
Wild-type (negative control)	C24	0	0

Table 2 Reverse phase high-performance liquid chromatography (RP-HPLC) elution and subsequent mass spectral analysis of human insulin (hIN) standard in comparison with trypsin-cleaved oleosin–human insulin (OB-hIN) collected from 17.0 to 17.5 min on a C18 column

Insulin	Sample	RP-HPLC retention (min)	Expected mass (Da)	Observed mass (Da)
hIN standard (Sigma)	hIN	17.213	5807.6	5807.6
OB-hIN trypsin-matured insulin	Plant-derived $DesB_{30}$-insulin	17.269	5706.5	5706.3

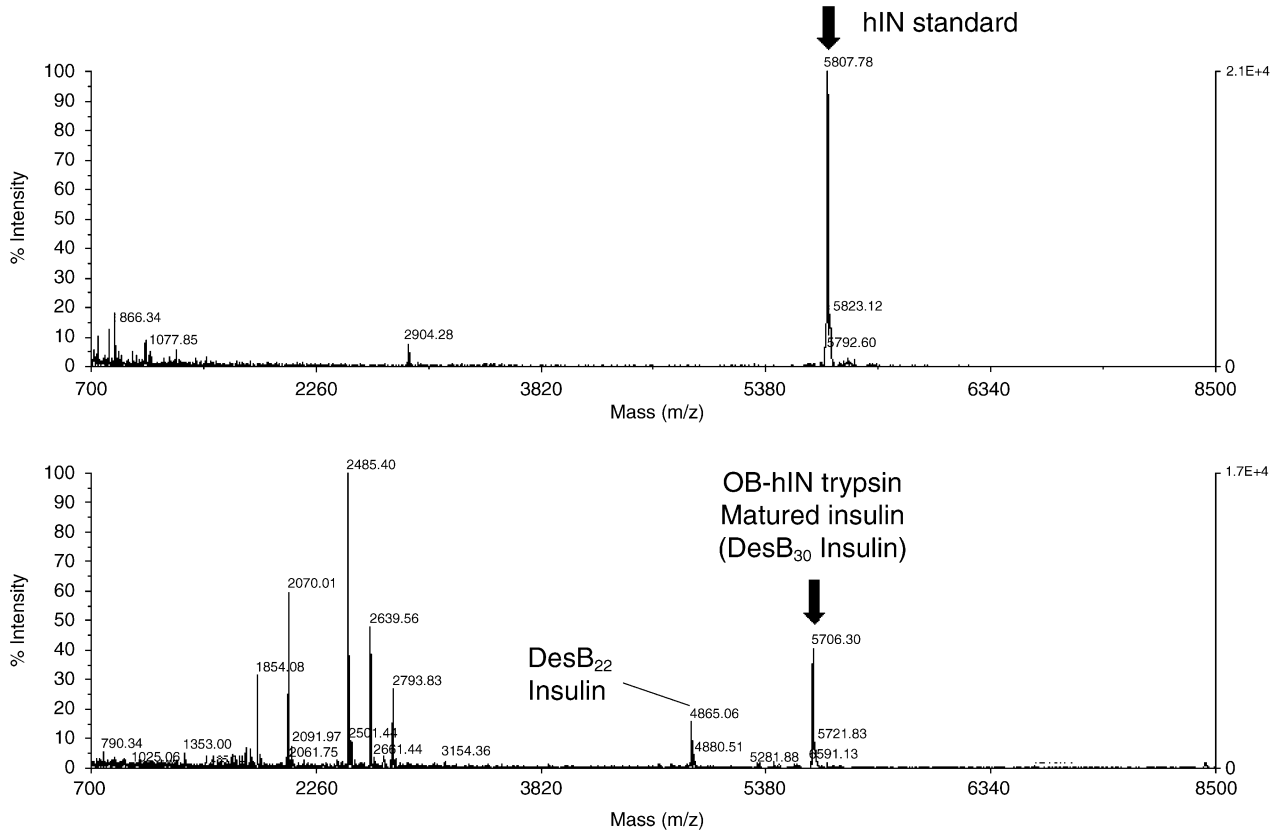


Figure 3 Mass spectral analysis of human insulin standard (hIN) in comparison with reverse phase high-performance liquid chromatography (RP-HPLC)-purified and trypsin-cleaved 4405 [oleosin–human insulin (OB-hIN) trypsin-matured insulin] fractions, collected from 17.0 to 17.5 min, as described in 'Experimental procedures'.

laser desorption/ionization time-of-flight (PSD MALDI/TOF) mass spectrometry, and the results confirmed that the mass of the product obtained following trypsin cleavage/maturation was identical to that predicted for authentic $DesB_{30}$-insulin (Figure 3, Table 2). In addition to the desired $DesB_{30}$-insulin, a number of product-related contaminants were resolved by mass spectral analysis. Based on the mass spectra, the product-related contaminants may include a

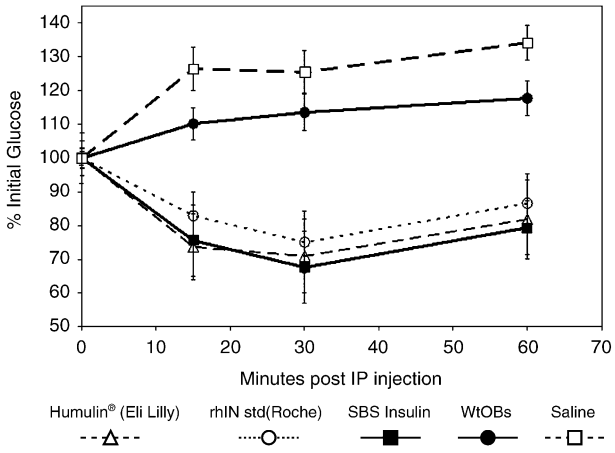


Figure 4 Temporal changes in serum glucose levels in male B6 mice following intraperitoneal (IP) injection of negative controls (open squares, saline placebo; filled circles, purified extract from non-transformed wild-type *Arabidopsis* seed oilbodies), positive controls (open circles, Roche hIN; open triangles, Humulin® Eli Lilly) and reverse phase high-performance liquid chromatography (RP-HPLC)-purified plant-derived $DesB_{30}$-insulin prepared from trypsin-matured oleosin–human insulin (OB-hIN) oilbodies (filled squares).

$DesB_{22}$-insulin. Apparently, these contaminants co-eluted with $DesB_{30}$-insulin during RP-HPLC purification and are most likely the result of incomplete cleavage or maturation with trypsin.

Plant-derived $DesB_{30}$-insulin exhibits biological activity

To confirm the biological activity of the seed-derived insulin, trypsin cleavage products from recombinant oilbody preparations were purified through anion exchange chromatography. The purified product was subsequently concentrated by lyophilization, re-suspended in 0.1 M HCl and quantified by enzyme-linked immunosorbent assay (ELISA) prior to assaying for activity. An insulin tolerance test using 2-month-old male C57Bl/6 (B6) mice was performed to demonstrate the functionality of plant-derived insulin *in vivo* (Figure 4). The results indicated that, at doses of 1 U/kg body weight, $DesB_{30}$-insulin was equally effective ($P < 0.05$) in lowering blood glucose as Humulin® and Roche insulin over the time course of the study. All insulins tested significantly reduced plasma glucose levels ($P < 0.05$) compared with controls of either saline or trypsin-treated wild-type *Arabidopsis* oilbodies. No toxic side-effects were observed with plant-derived $DesB_{30}$-insulin. Insulin purified from transgenic seed was also observed to induce autophosphorylation of the insulin receptor β-subunit (IRβ) of human hepatocellular carcinoma epithelial cells (HepG2) *in vitro*, as shown in Figure 5. This corroborates our findings obtained in the insulin tolerance test and suggests that $DesB_{30}$-insulin, purified and matured with trypsin from transgenic seed, is recognized by the insulin receptor and can elicit signal transduction. From our study, $DesB_{30}$-insulin appears to induce a more pronounced level of phosphorylation of the intracellular tyrosine kinase domains in comparison with the commercial insulin control. Potency measurements have previously shown that $DesB_{30}$-insulin exhibits a 116% relative activity (Moody *et al.*, 1974) to native insulin and this may account for the stronger response.

This proof-of-concept study was designed to assess the feasibility of the expression and recovery of biologically active human insulin in plant seeds employing SemBioSys Genetics Inc. technology. Results from this study were obtained in the model plant species *A. thaliana*, and demonstrated the utility of oilbody targeting and recovery technology for the production of human insulin in plants. Expression of insulin as an

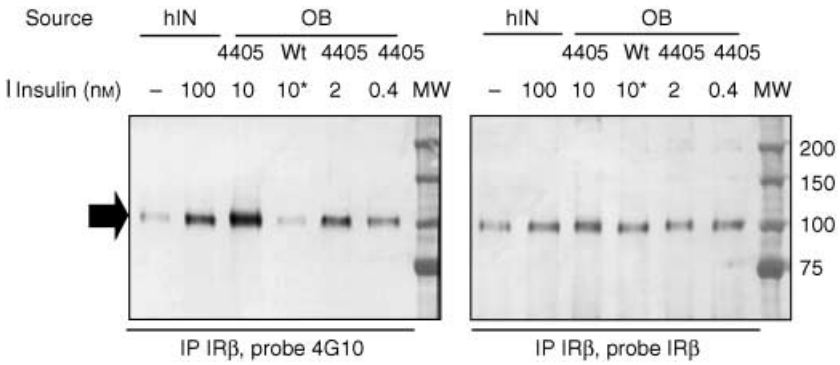


Figure 5 Western blots of cell lysates from HepG2 cells stimulated with various insulins (hIN, human recombinant insulin, Sigma; OB, $DesB_{30}$-insulin prepared from trypsin-matured oilbodies, 4405, or trypsin-matured non-recombinant wild-type oilbodies, Wt) at the concentrations listed. Cells were lysed, immunoprecipitated with an anti-insulin receptor antibody (α-IRβ) and probed with antiphosphotyrosine 4G10 (left) or α-IRβ (right). The arrow indicates the position of the insulin receptor (95 kDa). MW refers to the molecular weight markers of 200, 150, 100 and 75 kDa; 10* denotes the condition in which the equivalent amount of trypsin-matured, non-recombinant, wild-type oilbody processed material was used as a negative control; (–) denotes cells to which no insulin was added (commercial insulin-negative control).

oleosin fusion protein enables high levels of the recombinant protein to accumulate within the seed while, at the same time, providing for simplified downstream purification by flotation centrifugation (i.e. oilbody purification). Subsequent maturation to obtain a chemically authentic, biologically active product can be accomplished using standard enzymatic methods employed in the commercial manufacture of insulin from microbial systems.

The final phase of oilseed embryogenesis involves the process of desiccation, which results in a significant decrease in water content, and the seed enters a state of metabolic arrest (Nykiforuk and Johnson-Flanagan, 1998). During this physiological state of quiescence, stable accumulation of the recombinant protein in mature oilseeds affords a natural cellular 'warehouse' which can be stockpiled until recovery (maturation and purification) of the end-product is required to meet commercial demand and/or processing capacity. This provides a clear advantage over expression of recombinant protein targeted to sporophytic tissue which requires immediate purification of the product following harvest to avoid proteolytic degradation of the product. SemBioSys Genetics Inc. is currently assessing next generation constructs employing both Stratosome™ and Affinity Capture technologies in an effort to increase the overall expression levels of recombinant insulin in *Arabidopsis.* Concurrently, high-level expression constructs have been transformed into the commercial production platform crop safflower (*Carthamus tinctorius*) to provide for a high-capacity economical source of insulin capable of meeting future demand. Further to this end, product development efforts include the optimization of the maturation *in vitro* with trypsin and purification of the end-product from oilbody purified preparations.

Experimental procedures

Construction of pSBS4405:oleosin-Klip8-Klip27-MI fusion protein and transformation of *Arabidopsis*

The OB-hIN gene fusion was constructed by attaching a mini-insulin gene containing a trypsin-cleavable N-terminal synthetic propeptide sequence to the sequence encoding the 18-kDa oleosin from *A. thaliana*. The propeptide-mini-insulin (Klip27-MI) sequence was synthesized from four partially overlapping oligonucleotides which incorporated *A. thaliana* codon usage to increase the success of efficiency of translation in a plant-based expression system. Oligonucleotides (5′-GAAGAAGGAGAGCCTAAGTTTGTTAATCAACATCTTTGTGGATCTCATCTTGTTGAGGCTCTCTACCTTG-3′) and (5′-CCTTAGGAGTGTAGAAAAATCCTCTTTCTCCACACACAAGGTAGAGAGCCTCAACA-3′) were annealed at their complementary 20 nucleotide overlap and extended to form the 5′ end of the Klip27-MI fusion, whilst the same was performed with oligonucleotides (5′-CTAAGGCTGCTAAGGGAATTG-3′) and (5′-AAGCTTCAGTTGCAATAGTTCTCCAATTGGTAAAGTGAGCAAATAGAAGTGCAACATTGTTCAACAATTCCCTTAGCAGCCTT-3′) to form the 3′ end. The two halves were ligated following restriction digestion with *Bsu*36I to yield the full Klip27-MI coding sequence. Polymerase chain reaction (PCR) of this gene fusion using primers (5′-CTCGAGTCAACCAATTGATGACACTGAATC-3′) and (5′-AAGCTTCAGTTGCAATAGTTC-3′) attached a 5′ *Xho*I restriction endonuclease cleavage site and a 3′ *Hin*dIII cleavage site, respectively, for subsequent ligation in-frame with an existing *Arabidopsis* 18-kDa oleosin genomic sequence-Klip8 (Kuhnel *et al*., 2003) *Xho*I/*Hin*dIII site to create pSBS4405. The expression of the oleosin-Klip8-Klip27-MI transgene (OB-hIN) was under the tight control of the β-phaseolin promoter/terminator from *Phaseolus vulgaris* (Slightom *et al*., 1983). The phaseolin promoter controls the temporal- and tissue-specific expression of the transgene during seed development. Contained within the T-DNA of this vector is a *pat* gene conferring phosphinothricin resistance (Wohlleben *et al*., 1988) under the control of a ubiquitin promoter/terminator cassette from *Petroselinum crispum* (Kawalleck *et al*., 1993) in order to select positive transgenic plants. The complete expression cassettes between the right and left border of the T-DNA are annotated in Figure 1.

pSBS4405 was transformed into *E. coli* and subsequently into *Agrobacterium* (strain EHA101) using standard methods by electroporation (Van Rooijen and Moloney, 1995). The recombinant *Agrobacterium* was then used to transform *A. thaliana* (cv. C24) plants by the flower dipping method (Clough and Bent, 1998). The plants were allowed to grow to maturity and a mixture of seeds, untransformed and transformed, were harvested. For selection of transgenic lines, the putative transformed seeds were sterilized in a quick wash of 70% ethanol, and then 20% commercial bleach for 15 min, and then rinsed at least four times with double-distilled H_2O (ddH_2O). About 1000 sterilized seeds were mixed with 0.6% melted top agar and evenly spread on a half-strength MS plate (Murashige and Skoog, 1962) containing 0.3% sucrose and 80 μM of the herbicide phosphinothricin. After the establishment of roots, the putative transgenic seedlings were individually transferred to pots and allowed to grow to maturity. The pots were covered with a transparent plastic dome for 3 days to protect the sensitive seedlings. Positive seeds were then outgrown for another generation to generate T_3 seed and analysed.

Expression levels of insulin in *A. thaliana*

Expression levels of the OB-hIN fusion protein were determined in transgenic *A. thaliana* mature seed. For total seed extractable protein samples, 40 transgenic seeds were ground with a mortar and pestle in 50 μL of 50 mM Tris-HCl, pH 8.0. Reducing sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (6 × SDS sample buffer, 0.35 M Tris-HCl, pH 6.8, 30% glycerol, 10% SDS, 0.012% bromophenol blue, 5% β-mercaptoethanol) was then added to the slurry and mixed by briefly vortexing. The sample was then centrifuged and placed at 99 °C for 10 min. Samples were centrifuged, loaded (10 μL – equivalent to approximately seven seeds) and separated on discontinuous 15% SDS-PAGE gels. For negative controls, the same amount of wild-type C-24 *Arabidopsis* seed was prepared in the same manner.

For the preparation of oilbody samples, the transgenic and wild-type seeds (20 mg) were ground in 250 μL oilbody extraction buffer (0.4 M sucrose, 0.5 M NaCl, 50 mM Tris-HCl, pH 8.0). Samples were microfuged at 10 000 ***g*** for 10 min. The soluble aqueous fraction was removed with a 1-mL syringe and the fat pad was re-suspended in 100 μL of phosphate buffer supplemented with salt (20 mM Na_2HPO_4, pH 8.0, 0.5 M NaCl). The re-suspended fat pad was transferred to a clean microfuge tube and centrifuged again at 10 000 ***g*** for 10 min. The procedure was repeated three more times with a final re-suspension of the fat pad in 100 μL of phosphate buffer without salt (20 mM Na_2HPO_4, pH 8.0). An additional two more washes in phosphate buffer without salt were performed with intermittent centrifugation steps as outlined above. The final fat pad was re-suspended in 10 μL of phosphate buffer (20 mM Na_2HPO_4, pH 8.0). A 5-μL aliquot was taken and the oilbody protein was solubilized by boiling in 1/10 (v/v) 50 mM Tris-HCl, pH 8.0, with 2% SDS, and the protein content of the undernatant was determined by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) according to the manufacturer. For Coomassie-stained gels and Western blot analysis, 20 μg of total protein was separated on discontinuous 15% SDS-PAGE gels. Gels were subsequently Coomassie stained. The identity of OB-hIN in Coomassie-stained gels was established by blotting on to polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore Corporation, Bedford, MA, USA) for Western blot analysis. Blotted samples were probed with a monoclonal antibody directed against insulin (Clone E2E3 product ab9569) purchased from Abcam (Cambridge, MA, USA). Insulin bands were detected using a secondary sheep × mouse IgG F(ab')2 AP-conjugate (Chemicon International, Temecula, CA, USA) and developed using nitroblue tetrazolium–5-bromo-4-chloroindol-3-yl phosphate (NBT-BCIP) in GARAP buffer (Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

In vitro maturation with trypsin and subsequent HPLC purification

Prior to cleavage of the fusion protein (OB-hIN) with trypsin, the oilbodies were partially purified as follows. Approximately 1 g of transgenic seed was homogenized in 12 mL of extraction buffer (0.4 M sucrose, 0.5 M NaCl, 50 mM Tris-HCl, pH 8.0) and centrifuged at 10 000 ***g*** for 10 min; the fat pads were removed and placed in 1 mL of 50 mM Tris-HCl, pH 8.0, 0.5 M NaCl and re-centrifuged as above. This was repeated twice before washing and centrifuging the fat pad twice in 750 μL of 50 mM Tris-HCl, pH 8.0. The oilbody preparation results in the removal of the majority of background proteins.

The total protein content was determined by the BCA protein assay described above. Samples were then cleaved with trypsin (from bovine pancreas; Sigma-Aldrich, St Louis, MO, USA), at a 1 : 300 trypsin : total protein ratio, in 50 mM Tris-HCl, pH 8.0, on ice for 90 min to release the MI polypeptide from the fusion protein and remove the mini-C propeptide between the $DesB_{30}$ and A chains of the recombinant human insulin. The reactions were stopped with a 10-fold molar excess of *N-p*-tosyl-L-lysine chloromethyl ketone (TLCK; Sigma-Aldrich). Samples were centrifuged at 10 000 ***g*** for 10 min and the undernatants of the entire reactions were then filtered through 0.2-μm filters (Aerodisc® 13-mm Syringe filter with 0.2-μm Supof® membrane; Pall Corporation, Ann Arbor, MI, USA). The undernatants were further analysed by RP-HPLC using a C18 column (Zorbax 300SB-C18; Agilent Technologies, Waldbronn, Germany). Samples were loaded on to the column and eluted off at 1.0 mL/min using a 19-min linear gradient of 5%–50% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA). Fractions eluting at the same time as the insulin standard (yeast recombinant human insulin; Sigma-Aldrich) between 17.0 and 18.0 min were freeze dried and sent for mass spectral analysis.

Mass spectral analysis

PSD MALDI/TOF mass spectrometry was performed using a Voyager-DE STR mass spectrometer (Applied Biosystems, Foster City, CA). Mass spectral analysis was performed by the BioAnalytical Spectroscopy Service provided through NRC-Plant Biotechnology Institute, Saskatoon, SK, Canada.

Purification of trypsin-matured OB-hIN ($DesB_{30}$-insulin) using AKTA explorer

$DesB_{30}$-insulin was purified by anion exchange (Mono Q FF 1 mL; Amersham Pharmacia, Baie d'Urfe, QC, Canada) on an AKTA explorer (Amersham Pharmacia). Cleavage reactions were performed on oilbody preparations as described above, prepared from 30 g of transgenic seed. Samples were separated using a step gradient of 0–0.4 M NaCl with a 1 mL/min flow rate. Detection was performed at 214 nm (detection at 280 nm is relatively poor because of the low content of aromatic amino acids in insulin). Solvent A was 20 mM Tris-HCl, pH 6.5, while solvent B was 210 mM Tris-HCl, pH 6.5, 1.0 M NaCl. Fractions (1 mL) eluting at the same conductivity as Roche insulin standard (yeast recombinant human insulin; Roche Applied Science, Laval, QC, Canada), between 7 and 35 mS/cm, were collected and used in subsequent insulin tolerance tests. The presence of insulin was verified in the collected fractions by HPLC (as described above) and ELISA (Ultra Sensitive Rat Insulin ELISA kit; Crystal Chemical Inc., Downers Grove, IL). Samples collected were then concentrated by lyophilization and re-suspended in 0.1 M HCl prior to the insulin bioassay described below.

Insulin tolerance test: bioassay in male B6 mice

This bioassay was performed to determine the *in vivo* effect of recombinant plant-derived $DesB_{30}$-insulin in comparison with commercially available recombinant human insulins. Glucose plasma levels in B6 mice were determined prior to and following the intraperitoneal injection of insulin standards, negative controls and plant-derived $DesB_{30}$-insulin purified by RP-HPLC as described above. Fifteen male B6 mice, approximately 2 months of age, were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and were housed and fed *ad libitum* on a 12-h dark–light cycle. For insulin tolerance tests, mice were administered an intraperitoneal injection of insulin (at a dose of 1 U/kg body weight) and glucose levels were determined at 0, 15, 30 and 60 min using an automatic glucometer (OneTouch Ultra; Lifescan, Johnson and Johnson, Milpitas, CA, USA). Positive controls included Humulin® (Eli Lilly, Indianapolis, IN) and yeast recombinant human insulin standard from Roche (Roche Applied Science). A saline solution served as the placebo. A negative control was included: trypsin-cleaved oilbodies purified from wild-type (non-recombinant) *Arabidopsis* seed that was treated with trypsin and purified in the same manner as plant-derived insulin on the AKTA Explorer as described above. All insulin tolerance tests were performed on fed mice at the same time in the morning each day, with at least 2 days intervening between assays.

Insulin receptor autophosphorylation assay in HepG2 cells

Mammalian cell-based assays were performed to test the ability of plant-derived $DesB_{30}$-insulin to trigger insulin signal transduction through autophosphorylation of IRβ (White, 1998). HepG2 cells [human hepatocellular carcinoma epithelial cells, ATCC HB-8065; American Type Culture Collection (ATCC), Manassas, VA, USA] express insulin receptors and have been used previously to demonstrate insulin signalling (Frittitta *et al*., 2000; Senn *et al*., 2003).

HepG2 cells were grown at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Fresh medium was placed on the cells at least 18 h prior to the assay. Cells were removed from the plates with gentle scraping into fresh DMEM, and then stimulated with 100 nM commercial insulin or with 10, 5 or 2 nM plant-derived $DesB_{30}$-insulin for 10 min at room temperature. Cells were washed twice with 1 mL of cold phosphate-buffered saline (PBS) and then lysed in cold Nonidet P40 (NP-40) lysis buffer on ice for 10 min [50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES), pH 7.5, 150 mM NaCl, 1% NP-40, 10% glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulphonylfluoride (PMSF), 10 μg/mL leupeptin and 10 μg/mL aprotinin]. Insoluble material was removed by centrifugation at 10 000 ***g*** for 10 min. The insulin receptors were immunoprecipitated using an IRβ antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) followed by Protein A-Sepharose CL-4B (Amersham Pharmacia). Samples were washed three times in lysis buffer. Immunoprecipitated cell lysates were subjected to SDS-PAGE and blotted on to PVDF membrane (Immobilon-P; Millipore Corporation) for Western blot analysis as described above. Blots were subsequently probed with α-IRβ or antiphosphotyrosine 4G10 (Upstate, Waltham, MA, USA).

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