Final report for SFCCG

Effects of Plant Extracts on Insulin Production by Mouse Pancreatic Cells

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Abstract

There are currently 18.2 million people in the United States that are diabetic (Diabetes 2005). Their bodies inadequately produce or use insulin, a hormone that regulates the conversion of food and sugar into energy. More than half of diabetics prefer oral medications; hence further research involving alternative oral treatments is important for treating this disease. The overall purpose of this project is to investigate a mechanism by which alternative medicinal plants from the Democratic Republic of Congo (DRC) activate insulin response in a BTC-6 cell line derived from a mouse pancreatic beta cell that has stimulative properties to glucose. The purpose of this paper is to deliver essential techniques and findings during the initial stages of this research. Cells were fed 20 mL of medium every 48 hours and passaged weekly. Practiced techniques included 2D-electrophoresis and SDS PAGE, MALDI-TOF, use of the ImageMaster program and scanning of gels, protein purification, Bradford assay, and lysing of the cells. In the future, the aim of the study is to learn the mechanisms of action of different diabetes drugs. A drug from Kinshasa, Congo will initially be used. Extracts of plant species that have never been used scientifically, but have previously been used for medicinal purposes by Native Americans, will then be explored.

Introduction

Diabetes is a disease in which the body does not produce or insufficiently uses insulin, a hormone needed to regulate metabolism by turning sugars and other foods into energy needed for daily functioning (Diabetes 2005). Type 1 diabetes is when the immune system destroys the pancreatic beta cells, which are responsible for producing insulin. The most common type of diabetes is type 2, which affects 90-95% of diabetics. This is when the pancreatic beta cells eventually decrease insulin production due to the body’s efficient use of insulin (Matthews 2003). In the United States, there are currently 18.2 million people who are diabetic (Diabetes 2005). Out of these individuals, 5.2 million are unaware they have the disease. In addition, 41 million people are pre-diabetic (American 2005).
Common treatments used by diabetics to control blood glucose include changes in lifestyle, such as careful dieting, exercise, and losing excess weight. Insulin injections have been used by 19% of diagnosed adult diabetics, but taking oral medications is the most popular treatment, which 54% prefer. Since more than half of diabetics prefer oral medications, further research involving alternative treatments is important for treating the millions, and rising, diabetics across the country.

Sulfonylureas, which stimulate the pancreas to produce more insulin, have been the only type of pharmaceutical oral treatment available to type 2 diabetics until recently (Matthews 2003). Glipizide is an example of such a medication. An example of a common oral hypoglycemic agent is Avandia®. The drug not only makes the body more sensitive to insulin, but improves how the body uses insulin. Before the development of such pills however, plant therapies were documented to be beneficial in the treatment of diabetes (Gray and Flatt 1999). There are at least 400 plant species in the world that have been documented for being beneficial as alternate treatments to diabetes, but await evaluation and further research (Gray and Flatt 1999). For centuries, plants have been used for their medicinal properties. In the Democratic Republic of Congo, a compound from a fungus was found having properties similar to insulin. Since the compound is not a protein, it can bypass degradation, thus it can be taken orally unlike insulin injections (Zhang et al. 1999). The plant Coriandrium sativum (coriander) has been confirmed in previous studies to have had antihyperglycemic properties when added into the diets of streptozotocin-diabetic mice by increasing the secretion of insulin and/or improving the action of insulin (Gray and Flatt 1999). Coriander stimulated insulin secretion 1.3 to 5.7 fold from a pancreatic beta cell line. Also, 0.5 mM diazoxide was noted to inhibit this stimulation in the cell line (Gray and Flatt 1999).

The cell lines mentioned above have key attributes of normal islet cells thus make them useful in insulin research. The use of primary cells is limited due to difficulty in cell isolation as well as tissue supply (Hohmeier and Newgard 2004). In general, a pancreatic beta cell line is an endocrine cell that is grown in a culture and can process and secrete insulin (Poitout 1996). For example, the Beta Tumor Cell (BTC) lines are established by transgenic mouse offspring expressing SV40 (region controlled by rat insulin II gene promoter) in the pancreatic beta cells. Developed tumors are removed and propagated in a culture to make the cell line (Poitout et al. 1995 & 1996). These cells are grown at 37°C and fed fresh medium every other day. When confluent, the cells undergo passaging, which is when they are detached from the plate by being exposed to no more than 5 minutes of Trypsin/EDTA (Poitout 1996). The BTC-6 cell line has a 1.6 fold increase in insulin production upon glucose stimulus, where half the maximal response occurs at a glucose concentration of 0.5mM. When the BTC-6 cells are subjected to KRBB buffer at a 0mM [glucose] (basal), insulin secretion remains constant. However after exposure to 11.1 mM glucose and isobutylmethylxanthine (IBMX), there is a steeper jump in insulin secretion within a few minutes compared to secretion with 11.1 mM glucose and the absence of IBMX exposure(Graph 1). The response can also be enhanced by using glipizide (Hohmeier and Newgard 2004).
The mechanism of insulin secretion involves activation by cAMP and calcium signaling. Glucose and amino acids in the beta cells are the main stimuli of secretion and are controlled via depolarization and calcium channel activation (Horvath et al 1998). Overall, glucose enters the plasma membrane of a pancreatic beta cell by glucose transporters and is phosphorylated by glucokinase enzyme into glucose 6-phosphate, thus determining the rate of glycolysis. When blood glucose is high, the rate of ATP production increases and vice versa. Increasing the cytoplasmic ATP/ADP ratio closes K\textsubscript{ATP} channels, causing depolarization to occur in the plasma membrane. Voltage-dependent calcium channels open and a rapid flow of Ca\textsuperscript{2+} enter, triggering glucose-dependent insulin secretion (Doyle and Egan 2003). G-coupled pathway initiate phosphorylation and dephosphorylation protein reactions within the cell, thus regulating gene transcription linked to regulation of insulin secretion (see Diagram 1).
The overall purpose of this project is to investigate a mechanism by which alternative medicinal plants from the Democratic Republic of Congo (DRC) activate insulin response in mouse pancreatic beta cells. The purpose of this paper is to describe essential techniques and findings developed during the initial stages of this research. The future of this project will also be discussed.

**Materials and Methods**

Dr. Olsen from the University of Michigan had sent us a BTC-6 cell line derived from mouse pancreatic beta cells that have simulative properties to glucose. The following is a description of how the cells were treated. While growing these cells
through trial and error, techniques that will be later used on the cells were performed by
using other samples (e.g. commercial E.Coli and beef liver cells), not our own cell line.

Removal of DMSO from frozen cells

Frozen cells (stored at -80 °C) were warmed up to 37 °C and added to a well with
medium. The following day, that medium had to be removed in order to rid the cells of
the freezing agent, DMSO. The medium was centrifuged at 800g for 5 minutes. The tube
containing the pellet was carefully removed from the rotor, in order to not disturb the
pellet that formed. Medium containing the DMSO was removed with a pipette and stored
in a Petri dish. If the pellet dropped to the bottom, the tube was centrifuged again for 5
minutes. Fresh medium (2 mL) was added to the tube and transferred to an adjacent well.

Preparing the medium and feeding the cells

Medium was prepared by adding 50 mL Fetal Bovine Serum and 5 mL
Antibiotic/Antimycotic to 445 mL of stock RPMI 1640 medium. Medium was stored at
-4°C.

Cells in each of the 3 flasks were fed 20 mL of medium every other day and incubated
in the CO2 water jacket incubator. Prior to each feeding, medium was placed in a water
bath at 37°C for 30 minutes. The old medium from each flask was removed and
discarded.

Cell passaging

Medium, Dulbecco’s PBS, and trypsin were warmed up in the 37°C water bath.
Trypsin was not warmed up for a long time because it would self-digest. Clusters of cells
were checked by using the microscope. Medium was then removed from the flask. 10 mL
Dulbecco’s PBS was swirled in the flask, removed, and discarded. This was then repeated
in order to remove as much trypsin inhibitor that was in the medium as possible. 5 mL
Trypsin/EDTA was added, quickly removed until dry, and timed for approximately 5
minutes. During this time, detachment of the cell clusters from the surface inside the flask
was observed under the microscope. When few colonies detached, 11.5 mL of medium
was added. If a lot of detachment was observed, then 60 mL of medium would be added
to the flask. Medium was pipetted up and down several times inside the flask carefully in
order to avoid scratching the surface. The flask containing new medium was then
checked again under the microscope to observe dispersal of single cells. The 60 mL of
medium that was added to the flask was then split into different flasks for the next
passage. Passaging was done once a week for each flask.
**Cell counting and testing for cell viability after passaging**

50 µL of medium after passaging was pipetted onto a hemocytometer. Another 50 µL was pipetted into a test tube and trypan blue (450 µL) was added for staining. Under the microscope, colorless cells after the staining indicated viability. This medium/stain combination was placed on the hemocytometer as well. Cells were then counted and concentration (cells / mL) was determined. This was done by taking 4 of the grid boxes under the microscope and recording the number of cells visible per box. Then the total number of cells was found followed by an average count of cells per box. This number was then multiplied by 1.0 X 10^4 cells/ mL.

**Preparing the Krebs Ringer Bicarbonate buffer**

Krebs-Ringer Bicarbonate buffer containing no glucose (125 mM NaCl, 3 mM KCl, 1.2 mM MgSO4, 1.2 mM CaCl2, 22 mM NaHCO3, 1 mM Na H2PO4, 10 mM HEPES) was prepared and stored at room temperature (see Table 1 for amount of each reagent that was added based on molecular weights and concentration). The solution was titrated to a pH of 7.4 with NaOH. The volume was adjusted to 1L with deionized water. Half of the buffer was removed into a separate container and 0.1% BSA was added.

<table>
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<th>Reagent</th>
<th>mM</th>
<th>MW (g/mol)</th>
<th>g added</th>
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<tr>
<td>KCl</td>
<td>3</td>
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<tr>
<td>MgSO4</td>
<td>1.2</td>
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<td>1.2</td>
<td>110.9</td>
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<td>0.11998</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
<td>238.3</td>
<td>2.383</td>
</tr>
</tbody>
</table>

Table 1. Concentrations, molecular weights (g/mol), and amount added (g) for each reagent in the Krebs-Ringer Bicarbonate buffer.

**2D-Electrophoresis and SDS PAGE**

A rehydration/equilibration tray was placed on the lab bench. The commercial E. Coli sample was taken out of the starter kit (ReadyPrep™ 2-D starter kit, 2000). Readystrip 7cm IPG strips (pH 4-7) were removed from the freezer and placed on the lab bench to defreeze. 125 µl of the E. Coli protein sample was pipetted evenly (except for 1cm at each end) along channel #3 and repeated for channel #10. Forceps were used to peel the coversheet from 2 of the IPG strips. Strips were places gel side down into the
channels. The + and pH 4-7 end was on the left side of the channel. Mineral oil (1mL) was added on top of the strip to prevent evaporation. The tray was covered and left on the lab bench overnight.

The following day, the protean IEF focusing tray was placed on the lab bench. The tray was checked for oil residue. If oil was still in channels, the tray was washed again and dried. Paper wicks were placed on the vertical metal electrodes on each end of the channels used (e.g. channels 5 and 8). Nanowater (8 µL) was pipetted on top of each wick. The mineral oil was then drained off the IPG strips by holding the strip vertically for approximately 8 seconds with the forceps over the rehydration/equilibration tray. The IPG strips were transferred into the focusing tray maintaining the gel side down. Mineral oil (1 mL) was added on top of each IPG strip. The lid was placed on the tray (+ on the left) and placed into the PROTEAN IEF cell. The instrument was closed and the appropriate stored program was chosen (<Stored tests/<Ecoli-S3/< # of Gels: enter 1). START was pressed to begin the electrophoresis run. For the 7cm strips, the estimated run time was 5 hours (14,000 volts per hour).

After the test was run, the tray was taken out of the instrument. IPG strips were removed from tray and drained of mineral oil by being held vertically for ~ 8 seconds. Each strip was placed on a piece of dry blotting paper gel side up. Another piece of blotting paper that was dampened with nanopure water (~ 1mL) was placed on top of the gel strip and gently pressed down. The blotting paper was slowly peeled off the gel from one end to the other. Oil-free strips were transferred to fresh, dry channels in the rehydration/ equilibration tray gel side up. To see if isoelectric focusing worked, one strip was stained in a staining tray containing a few mls of Coomassie Blue stain and rocked for 1 hour maximum. The stain was removed and destained 2 times for 10 minutes each.

An 8-16% precast polyacrimide gel was taken out from the refrigerator and removed from its packaging. The IPG comb was extracted from the gel and the well was rinsed with nanopure water using a pipette. Equilibration buffer 1 (containing 30% glycerol) and Equilibration buffer II (containing 30% glycerol and iodoacetamide) were removed from the kit and each bottle was warmed slightly with hands until the solids liquefied. 2.5 mL of equilibration 1 buffer was pipetted on top of the remaining IPG strip in the rehydration/equilibration tray. The tray was rocked for 10 minutes on the orbital shaker. The equilibration 1 buffer was then drained from the tray, and 2.5 mL of equilibration 2 buffer was added. The tray was returned to the orbital shaker for another 10 minutes and then the buffer was discarded.

Agarose gel was removed from the kit and melted in the microwave for 45-60 seconds and pipetted into the well. The IPG strip was dipped briefly into 10 mL of 1X Tris/glycine/TGS running buffer. With gel side up, the strip was laid down in the IPG well (+ and pH 4-7 on left) and pressed down into the well containing agarose with forceps. The polyacrimide gel was mounted in the electrophoresis apparatus (facing inward) and the reservoir was filled with running buffer. SDS-PAGE was run for 40 minutes at 200V. To make sure the voltage was running through, bubbles were looked for inside the apparatus. After the gel was run, the gel was removed from the apparatus and put in a staining tray containing Coomassie Blue stain and rocked overnight. The following day the gel was removed from the rocker and the stain was put back into the bottle it came from. Destainer was then added (95% MeOH, 10% acetic acid, and 95% deionized water).
MALDI-TOF

In order to see how MALDI-TOF works, 250 µL of 30% Acetonitrile, 70% H2O and 0.1TFA was added to Sinapinic acid. Sinapinic solution (20µL) was added to 1µL Tryptic digest of BSA (500 pmol), 1 µL egg albumin (219.19 µM) and 1µL glycerol 3-P dehydrogenase (27.6 µM) that were in separate vials. The anchor chip target plate was spotted with 1 µL of each solution and the target spot for each sample was recorded (e.g. H5: Tryptic Digest of BSA, H6: albumin egg, etc).

The computer and monitor attached to the mass spectrometer were turned on. The Log In name was already entered, but the password needed to be typed in. FLEX CONTROL was opened in order to open the software. CHOOSE APPROPRIATE METHOD was clicked on and 66KDA method was chosen. The spots of each sample on the target plate were checked to see if they were completely dry before the plate entered the mass spectrometer. The Load/Eject button on the mass spectrometer was pushed and A1 on the target plate was aligned with the A1 inside the instrument. Load/Eject was pressed again.

On the computer, the target was clicked on (e.g. when we wanted to analyze albumin we would click on the target dot H6) then START was clicked on to get the spectrum. The power notch was increased if there was a lot of noise and START was clicked again. The spectrum was saved to file by first clicking on DISPLAY LABELED PEAKS to get a label on each peak. The Green tube on the tool bar was then clicked for Bioflex analysis to pop up. When in the window for mass spectrum, EDIT MASS LIST on the tool bar was clicked. The left of each peak was clicked on in order to add each peak to the mass list. To change the X scale of the spectrum, ZOOM IN X RANGE was used. TOOLS was then clicked on, followed by BIOTOOLS to transfer our spectrum pattern to the database for identification of the protein. Username and email were typed in and submitted. From the Blast results, the identification with the highest match was found for the targeted sample.

ImageMaster Program and Scanning of Gels

The gel obtained after running 2D electrophoresis and SDS page was scanned into the Imagemaster 2D platinum computer program. First the scanner was turned on. After opening the program, we went to ‘import→twain→acquire’ then scanned the gel. This program was used for quantification analysis of the proteins on a gel. To obtain tutorials on how this program worked, we went to ‘Help’ then ‘Tutorials.’ Tutorial 1 dealt with experimental setup, whereas tutorial 2 was about viewing and manipulating gels. Tutorial 3 encompassed learning about matching gels. Tutorial 4 focused on data analysis.
**Protein Purification**

Beef liver protein (500 µg) was transferred to a final volume of 100 µL into a 1.5 mL microcentrifuge tube (ReadyPrep™ 2-D cleanup kit, 2000). 300 µL of precipitating agent 1 was added to the protein sample and vortexed. The sample was incubated for 15 minutes in an ice bath. 300 µL of precipitating agent 2 was added to the mixture and vortexed again. The tube was centrifuged at maximum speed for 5 minutes to form a tight pellet. Once removed from the rotor, the supernatant was removed using a pipette and discarded. The pellet was centrifuged once more for 30 seconds in order to remove the remaining supernatant from the tube. 40 µL of wash reagent 1 was added on top of the pellet and centrifuged for 5 minutes. The wash was then removed and discarded. 25 µL of nanopure water was added on top of the pellet then vortexed for 20 seconds. 1 mL of wash reagent 2 and 5 µL of wash 2 additive was added and vortexed for 1 minute. The protein-containing solution was incubated at -20°C for 30 minutes, where every 10 minutes the tube was removed from the freezer and vortexes for 30 seconds. The tube was centrifuged for 5 minutes after incubation, followed by removal of and discarding the supernatant. The tube underwent another 30 seconds of centrifugation and the remaining wash was discarded. The pellet was air-dried for less than 5 minutes and 370 µL of 2D rehydration sample buffer was added to resuspend the pellet (appropriate volume of buffer was determined based on a proportion : 125 µL E.Coli sample volume used previously/169 µg protein loaded = __ µL our protein sample volume / 500 µg protein loaded). The tube was vortexed for 30 seconds and incubated at room temperature for approximately 4 minutes. The tube was vortexed once more for a minute and solution was pipetted up and down for complete suspension of the pellet. The solution was centrifuged once more for 5 minutes and the supernatant was used for 2D-electrophoresis.

**Bradford assay and lysing of cells**

A standard curve was graphed based on absorbances of increasing concentration of BSA (0, 0.2, 0.4, 0.6, 0.8, and 1.0 µg/mL) using a spectrophotometer (595 nm). 0.2 mL of all solutions that were prepared was distributed into new tubes and 1 mL of Bradford reagent was added to each. Each tube was vortexed and incubated for 5 minutes. Solutions were measured for absorbances starting with a blank and measuring in order of increasing depth of color.

Beef liver cells (0.209 g) in 2 mL of 10mM sodium phosphate buffer was lysed with a sonicator power 3 cycle for 1 minute. The lysed cells-buffer solution was briefly centrifuged at maximum speed (>12,000 X g). The supernatant was pipetted out and saved, whereas the pellet was discarded. Bradford reagent (967 µL) was added to 33 µL of the supernatant. In case the absorbance was too high for the standard curve, the supernatant was diluted 10 fold (3.3 µL supernatant and 29 µL buffer). Protein concentration of supernatant was determined based on the known absorbance and the standard curve generated (Graph 2 in results and discussion).
Results and Discussion

Removal of DMSO, preparing the medium, and feeding the cells

Prior to feeding, the conditions of the cells were recorded for each of the 3 flasks we had. We only had 3 flasks because the other 6 became contaminated. Despite this significant loss of cells, clusters of cells were observed and healthy round cells were visible for P44 on May 17, 2005. Clusters are a sign of progress because it shows the cells have grown and amplified in numbers. If cells are not clustered then there is no need for passaging. However, over the following month, such progress in cell growth diminished. When the 3 flasks of cells were all mainly dead, we took out our backup of frozen cells that was stored at -80 °C, removed the DMSO, and started all over, while saving whatever we could from the flasks. The cell density of the defreezed cells was 54.5 X 10^4 cells/mL. We found out that cells have to be grown in smaller flasks at high densities. Pancreatic beta cells grow better when in close proximity for communication with each other. In a large flask, like the ones we were using, the cells were more spread out and growth was insufficient for them over time. To overcome this problem, we transferred the cells from the only P44 flask containing some viable cells into one of the wells on a 6 well plate and also began growing the new cells in the wells at a higher density in a separate well.

From mid-May to early June, the color of the fresh medium was initially orange-red, but would turn into a magenta color after a couple days. Dr. Olsen suggested that the reason our cells were dying was from lack of oxygen. We disputed that, as we discovered that inadequate CO_2 concentration was the likely cause of it. It was also brought to our attention that antibiotic-antimycotic should be avoided in the medium for our BTC-6 cell line. This could be another reason our cells were not growing and barely surviving.

Cell passaging and cell counting

Break up of clusters should have formed healthy and well-rounded single cells. There was some detachment, since some cells were floating on the surface, but this took 15 minutes to see after being washed with trypsin. When the single cells did not form, this was either because trypsin was not as active as it used to be or the cells were dead. We decided to proceed with whatever was viable, feeding the new passage 11.5 mL of medium. If we had a lot of cells detached, then we would have used 60 mL to dilute the cells. The medium was then pipetted up and down to break clusters into single cells. When looking under the microscope again, there were some single cells floating but clusters were still attached, indicating the Trypsin/EDTA vial we used was not effective. It was later brought to our attention by Dr. Olsen that we have to trypsinate the cells for at least 15 minutes. It can also take up to 3 days to break up clusters.

Approximately 50 mL of medium from P44 was extracted and added to the hemocytometer. The cell density was 3.70 X 10^4 cells/mL. This is not a high cell density at all considering this number is not even a half a million cells. Hence we did not have much cell growth. The cells were also not as round and healthy, but rather dehydrated and jagged. When the stained medium was pipetted onto the hemocytometer, the majority of the cells were dark, indicating they were dead. This flask was therefore bleached and
discarded. The other flasks did not have enough cells for passaging. Normally, if we had added 60 mL of medium to a flask containing a high density of single and healthy detached cells, we would split it into different bottles for the next passage.

*ImageMaster Program and Scanning of Gels from 2D-Electrophoresis and SDS PAGE*

After 2D-electrophoresis was run on the commercial BSA, the stained IPG strip showed distinct bands. These bands represent protein separation by current based on their pI values, indicating that Isoelectric Focusing was successful. The gel after running SDS PAGE is in Figure 2 and 4. The red crosses in figure 4 are indicators of protein presence, a technique available on Imagemaster. The dashed red box in figure 2 is the area of the gel used to obtain a 3-D image of the proteins (Figure 3). The height of each peak corresponds with the protein intensity. Due to time constraints, protein concentration values based on intensity could not be obtained.

Figure 2: SDS Page gel of commercial BSA

Figure 3: 3D view of the protein intensities in the red dotted area of figure 2.
MALDI-TOF

Because the protein samples we ran were identified as Tryptic digest of BSA, Egg albumin, and glycerol 2-P dehydrogen prior to MALDI-TOF, there was no need to record the blast results in this particular case.

Bradford assay and lysing of cells

The absorbance of supernatant diluted 10 fold [3.3 µL stock supernatant containing the lysed beef liver cells with 29 µL buffer (10 mM sodium phosphate) was 0.590 A. From the line equation of the standard curve in graph 1, the protein concentration of the diluted supernatant was 0.813 mg/mL. Hence the concentration of protein in the stock supernatant, 8.13 mg/mL, was found using the Bradford assay.

Protein Purification

Once the beef liver protein was purified, 2D electrophoresis and SDS page was performed on the supernatant. However, the SDS PAGE gel came back blank so either the isoelectric focusing or SDS PAGE were unsuccessful. We should have added a larger volume of 2D rehydration sample buffer to the suspended pellet. Then we would have had enough supernatant to run 2 IEF strips and one could have been stained. Instead, we only made enough supernatant for running the gel. Therefore we could not determine if the failure to obtain the gel was because of a problem with IEF or SDS.
Conclusion

Even though the CO₂/water jacket incubator was contaminated in May, the cells were successfully passaged three times before they were destroyed by mold. We were able to learn by trial and error and from insight provided by Dr. Olsen (University of Michigan) what to avoid in the future regarding cell treatment.

In the future, the aim of the study is to learn the mechanisms of action of different diabetes drugs. First, a drug from Kinshasa, Congo will be used, followed by the extracts of plant species that have never been explored scientifically, but have previously been used for medicinal purposes by Native Americans. For the 18 million, and rising, diabetics in the United States alone, we hope this project leads to an alternative drug to help treat the disease.
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