Original Article

Lupin gamma conglutin protein: effect on Slc2a2, Gck and Pdx-1 gene expression and GLUT2 levels in diabetic rats


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A B S T R A C T
Recently, lupin seed (Lupinus albus L., Fabaceae) products have emerged as a functional food due to their nutritional and health benefits. Numerous reports have demonstrated the hypoglycemic effects of lupin’s gamma conglutin protein; nonetheless, its mechanism of action remains elusive. To understand the role of this protein on glucose metabolism, we evaluated the effect of administering L. albus gamma conglutin on Slc2a2, Gck, and Pdx-1 gene expression as well as GLUT2 protein tissue levels in streptozotocin-induced diabetic rats. While consuming their regular diet, animals received a daily gamma conglutin dose (120 mg/kg per body weight) for seven consecutive days. Serum glucose levels were measured at the beginning and at the end of the experimental period. At the end of the trial, we quantified gene expression in pancreatic and hepatic tissues as well as GLUT2 immunopositivity in Langerhans islets. Gamma conglutin administration lowered serum glucose concentration by 17.7%, slightly increased Slc2a2 and Pdx-1 mRNA levels in pancreas, up-regulated Slc2a2 expression in the liver, but it had no effect on hepatic Gck expression. After gamma conglutin administration, GLUT2 immunopositivity in Langerhans islets of diabetic animals resembled that of healthy rats. In conclusion, our results indicate that gamma conglutin up-regulates Slc2a2 gene expression in liver and normalizes GLUT2 protein content in pancreas of streptozotocin-induced rats.

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Introduction

In recent years, consumers interest in plant-based foods, as well as foods with high protein content, has significantly increased. Although protein intake in some countries exceeds the daily requirements, consumption of specific protein sources may be beneficial for some segments of the population.

In this sense, the genus Lupinus is a legume constituted by many species distributed worldwide. Interestingly, its seeds have a high protein content (35–40% dry basis). Lupin seeds are traditionally consumed in some areas for their purported beneficial effects, especially the species L. albus L., Fabaceae, whose properties have been widely described (Duranti et al., 2008; Arnoldi et al., 2015; Lucas et al., 2015).

In addition to globulin and albumin proteins, lupin seeds contain other bioactive compounds including alkaloids, toco-pherols, carotenoids, and polyphenols (Lampart-Szczapa et al., 2003; Ganzera et al., 2010; Arnoldi et al., 2015). Lupin globulins reduce circulating and tissue lipids as well as glucose levels (Brandsch et al., 2010; Radtke et al., 2015). More specifically, lupin’s gamma conglutin (Cy) protein reduces serum glucose levels in both diabetes- and insulin resistance-induced rats (Vargas-Guerrero et al., 2014; Gonzalez-Santiago et al., 2017). In healthy animals, Cy attenuates the glycemic peak after a glucose overload similar to the pharmacological agent metformin (Magni et al., 2004).

Alterations in carbohydrate and lipid metabolism are critical in the development of metabolic diseases such as diabetes and its complications (JDF, 2017; ADA, 2017). Type 2 diabetes is characterized by persistent hyperglycemia, resulting from either defective...
insulin secretion, impaired insulin action, or both (Ozougwu et al., 2013).

The pancreatic and duodenal homeobox 1 (Pdx-1) is a major transcription factor that regulates pancreatic β-cells differentiation, development, and function. The targets modulated by Pdx-1 include the solute carrier family 2 member 2 (Slc2a2/Glut2) and glucokinase (Gck). Both Slc2a2 and Gck play essential roles in glucose transport, insulin secretion, and glucose homeostasis (Wataba et al., 1996; Waeger et al., 1996; Albgren et al., 1998; McKinnon and Docherty, 2001; Fujimoto and Polonsky, 2009; Pedica et al., 2014). An elevation in circulating glucose levels triggers the insulin secretion by β-cells. This process requires glucose internalization by GLUT2 and its subsequent phosphorylation by GCK (Rorsman and Renstrom, 2003).

Previously, we have shown that the administration of L. albus' Cγ moderately increases insulin content and Ins-1 gene expression in the pancreatic tissue of experimentally-induced diabetic rats (Vargas-Guerrero et al., 2014). However, we were not able to identify reports regarding the effect of Cγ on Pdx-1, Slc2a2, or Gck pancreatic gene expression.

GLUT2 and glucokinase, encoded by the Slc2a2 and Gck genes respectively, play a significant role in hepatic glucose metabolism by promoting glycogen synthesis and lipogenesis. Therefore, diseases like diabetes show altered Slc2a2 and Gck expression (Jyndjian et al., 1988; Yonamine et al., 2016). Interestingly, Cγ promotes higher glucose uptake in HepG2 cells (Lovati et al., 2012; Capraro et al., 2013), indicating that this protein might up-regulate the Slc2a2 and Gck genes.

In previous work, we also investigated the effect of Cγ on the gluconeogenic gene expression (glucose-6-phosphatase, G6pc; fructose-bisphosphatase 1, Fbp1; and phosphoenolpyruvate carboxykinase 1, Pck1) and found that Cγ negatively regulates G6pc gene expression and lowers hepatic glucose production (Gonzalez-Santiago et al., 2017). However, the effect of Cγ on Slc2a2 and Gck gene expression in the liver of diabetes-induced rats has not been proven.

Although there have been advances in elucidating the Cγ mechanism of action, additional data is needed before it can be fully understood. Consequently, this study aimed to evaluate the effect of Cγ protein from L. albus on Slc2a2 and Pdx-1 mRNA and GLUT2 protein content in pancreas as well as Slc2a2 and Gck mRNA content in liver of rats with streptozotocin-induced diabetes.

Material and methods

Experimental design and animal group assignment

Male Wistar rats, supplied by the University of Guadalajara Bioterium, were housed in individual cages and placed in an air-conditioned room (24 ± 2 °C) and 55.0 ± 5% relative humidity with a 12 h light–dark cycle. Animals had free access to a standard rodent diet (LabDiet, PMI Nutrition International, St. Louis, MO, USA) and water. All experimental animal procedures were approved by the University of Guadalajara Bioethics Committee (C1.023/2014) and adhered to the International Guidelines for Care and Use of Laboratory Animals.

Experimental animals were randomly allocated into one of three groups of five rats each as follows: (1) healthy control group (Ctrl), (2) diabetic group without treatment (STZa), and (3) diabetic Cγ-treated group (STZa-Cγ). The Ctrl and STZa groups received 1.5 ml of carrier solution (0.9% w/v NaCl) while the STZa-Cγ group received Cγ (120 mg/kg BW) dissolved in carrier solution. Treatment solutions were administered by oral gavage once a day for seven consecutive days and serum glucose levels measured at the beginning and at the end of the treatment period. After sacrificing the animals, we collected their pancreas and liver to quantify gene expression and GLUT2 protein tissue content.

STZ-induced diabetes

Rats with a fasting glucose levels ≥200 mg/dl 72 h after the streptozotocin (STZ) injection were deemed diabetic and included in the study. Diabetes was chemically-induced in male Wistar rats (200–250 g) by a single intraperitoneal (i.p.) injection of STZ (65 mg/kg BW) (Sigma, St. Louis, MO, USA) dissolved in 0.1 M sodium acetate buffer, pH 4.5.

Plant material

Dr. Edzard van Santen from the College of Agriculture, Auburn University, Alabama kindly provided the dry, certified lupinus albus L., Fabaceae, seeds used in this study.

Extraction and validation of Cγ isolation

Cγ was isolated from hexane defatted lupin flour as previously described (Vargas-Guerrero et al., 2014). Briefly, albumins were extracted twice with double distilled water (DDW) at 4 °C under constant stirring for 2 h and 1:10 (w/v) flour to water ratio. After each extraction, the mixture was centrifuged for 30 min at 10,370 × g at 4 °C, and the supernatant discarded. Subsequently, the pellet was suspended in 10% NaCl (pH 7.0), the solution stirred for 12 h at 4 °C, it was centrifuged for 30 min at 4 °C and 10,370 × g, and finally the supernatant was saved. The globulins fraction, recovered from the supernatant by precipitation with 85% ammonium sulfate, was dissolved in 0.1 M phosphate buffer and dialyzed against 0.2 M acetate buffer (pH 4.8) for 18 h at 4 °C. The retentate was then centrifuged to separate α-conglutin (in the pellet) from β- and γ-conglutin (supernatant). Finally, the supernatant was dialyzed against DDW for 48 h at 4 °C and the retentate containing Cγ centrifuged. The resulting supernatant solution, containing Cγ, was lyophilized (Freeze Zone, LABCONCO) at −50 °C, 0.036 mbar for 8 h.

The presence of Cγ in the isolate fraction (2 mg per sample) was verified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 12% under reducing and non-reducing conditions using a mini Protein® Tetra cell (BioRad, Milan, Italy) equipment (Schagger and von Jagow, 1987). Gels were stained after electrophoresis with Coomassie brilliant blue G-250 (BioRad, Milan, Italy). The relative molecular weight of native and denatured Cγ was determined by comparison with a protein ladder (BenchMark™ Prestained protein ladder, Invitrogen).

Serum collection and glucose quantification

After an overnight fasting period, blood was collected from the retro-orbital plexus of sedated animals and centrifuged for 15 min at 6000 × g and 4 °C to separate the serum. Serum glucose concentration (mg/dl) was quantified using the glucose oxidase-peroxidase reagent (BioSystems, Spain) and a spectrophotometer analyzer (BTS 350, BioSystems, Spain). Animals were anesthetized with a single dose of 80 mg/kg BW of tiletamine-zolazepam (Zoletil® 50; Virbac, Carros, France).

GLUT2 immunopositivity in β-cells

Immunohistochemistry established GLUT2 immunopositivity in pancreatic β-cells. The excised pancreas from each animal was washed with saline solution, immediately fixed in 4% p-formaldehyde and embedded in paraffin. Four μm thick sections were dewaxed at 62 °C and the tissue rehydrated with graded concentrations of ethanol (10 min) followed by a 10 min incubation in...
citrate buffer (pH 6.0) and a 60 min incubation in 5% fetal bovine serum (FBS).

Tissues were later incubated at 4°C overnight with the GLUT2 primary antibody (Anti-Glucose Transporter GLUT2 antibody Abcam ab 54460, Cambridge, USA) at a 1:100 dilution and rinsed three times for 2 min each with 1 x TBST. Mouse/Rabbit Immunodetector HRP/DAB Detection System (BIO SB, USA) was used to reveal the GLUT2-bounded primary antibody. 3,’3’-Diaminobenzidine (DAB) was added to the tissue and incubated at room temperature for 20 min in the dark, and then washed for 5 min with DDW and stained for 5 min with hematoxylin. For negative controls, we replaced the primary antibody with 1 x phosphate buffer saline (PBS).

The immunopositive GLUT2 regions were determined by detecting and quantifying the DAB intensity with Motic Images Plus 2.0 software (Motic China Group Co. Ltd., China). The total and GLUT2-positive average areas were calculated for each sample in the experimental groups, and the percent of GLUT2 immunopositivity calculated as follows:

\[
\text{GLUT2 immunopositivity} \% = \left( \frac{\text{Average GLUT2 positive area}}{\text{Average total area}} \right) \times 100
\]

RNA was isolated from hepatic and pancreatic tissues and reverse-transcribed into cDNA as previously described (Gonzalez-Santiago et al., 2017). Afterwards, we quantified Slc2a2 (pancreas and liver), Gck (liver) and Pdx-1 (pancreas) gene expression by quantitative real-time PCR (qPCR). The amplification involved an initial denaturation process (95°C for 10 min), followed by 45 cycles comprised of a denaturation step (95°C), annealing and elongation (72°C) cycles. Table 1 shows the primer sequences, number of the UPL probes, and annealing temperature for each gene.

For the Slc2a2, Pdx-1, and Rps18 gene expression, we used the LightCycler TaqMan Master Mix (Roche, Germany) and Universal Probes Library (UPL, Roche, Germany). To quantify the Gck gene expression, we used a LightCycler® FastStart DNA MasterPLUS SYBR Green I Kit (Roche).

Quantification of the gene expression of all reactions was determined in triplicate using a LightCycler 1.5 Instrument® (Roche Applied Science, Mannheim, Germany). For negative controls, sterile water replaced the cDNA. The Rps18 housekeeping gene served as an internal control. We used the 2\(^{−ΔΔCt}\) method for the relative quantification of Slc2a2, Pdx-1, and Gck gene expression. The crossing threshold (Ct) values obtained for the target gene were normalized against Rps18 Ct values.

### Glycogen content in hepatic tissue

Hepatic tissues were fixed in 4% p-formaldehyde and embedded in paraffin. Four-μm sections were mounted on slides and hydrated in bidistilled water. After, the slides were incubated in 0.5% periodic acid solution for 10 min, stained with Schiff’s reagent for 15 min and counterstained with hematoxylin solution for 2 min. The periodic acid-Schiff (PAS) positive sections were interpreted as percentage and cellular distribution of glycogen.

### Data analysis

Data analysis was carried out using PASW Statistics v. 18 software (IBM Corp., Chicago, IL, USA). We computed the average serum glucose levels (pre- and post-treatment) and the standard error of the mean (SEM) for each group. We also calculated GLUT2 immunopositivity, as a percentage, for each Langerhans islet analyzed and the Slc2a2, Pdx-1, and Gck gene expression, as relative units (RU) ± SEM. The Wilcoxon test established significant differences between pre- and post-treatment serum glucose levels within each group. The Mann-Whitney U test assessed statistical significance for gene expression, and immunopositivity to GLUT2. p values <0.05 were deemed statistically significant.

### Results and discussion

The effect of Cy on some molecules related to metabolic pathways has been described in previous works (Terruzzi et al., 2011; Lovati et al., 2012; Vargas-Guerrero et al., 2014; Gonzalez-Santiago et al., 2017). However, to further understand the mechanism of action of Cy, more studies are required. Here, we determined the effect of L. albus’ Cy protein on Slc2a2, Gck, and Pdx-1 gene expression as well as GLUT2 protein tissue content under diabetic conditions.

We isolated Cy from defatted lupin flour. After that, we verified its purity by SDS-PAGE under both, reducing and non-reducing conditions. As previously reported, a typical single ~49 kDa band corresponding to the native protein was found under non-reducing conditions whereas two bands (~17 and ~25 kDa) were present under reducing conditions: thus, confirming the presence of Cy (data not shown) (Vargas-Guerrero et al., 2014).

Subsequently, we validated the STZ-induced diabetes model. As expected, most animals developed marked hyperglycemia after a single STZ i.p. dose. Experimental animals with fasting glyceremia ≥200 mg/dl were deemed diabetic and included in the study.

### Cy treatment diminishes serum glucose levels in diabetic animals

In Fig. 1, the serum glucose levels (pre- and post-treatment) and the SEM for the three experimental groups are shown. After the seven-day treatment period, there was no significant change in glycemia in the Ctrl group (p > 0.05).

Conversely, serum glucose level increased by 30.5% in the STZa group, but significantly decreased (~17.7%) with the daily Cy administration (120 mg/kg BW). These findings are in agreement with results from previous work showing that Cy intake lowers glucose levels under hyperglycemic conditions (Lovati et al., 2012; Vargas-Guerrero et al., 2014). Glucose uptake by peripheral tissues is relevant to preserve normoglycemia, especially during postprandial state (Alvim et al., 2015; Gannon et al., 2015).

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>UPL number</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>Slc2a2</td>
<td>122</td>
<td>5′-AAAGCCCCAGATACCTTACT-3′</td>
<td>5′-GGCCCCATAGCTTTTTCAAGC-3′</td>
<td>60 C</td>
</tr>
<tr>
<td>Pdx-1</td>
<td>95</td>
<td>5′-AAAGCCCCAGATACCTTACT-3′</td>
<td>5′-GGCCCCATAGCTTTTTCAAGC-3′</td>
<td>60 C</td>
</tr>
<tr>
<td>Rps18</td>
<td>1</td>
<td>5′-CTCTAACGGGAGCAAGAGG-3′</td>
<td>5′-GGGATTTTGGGAGACGGGA-3′</td>
<td>63 C</td>
</tr>
</tbody>
</table>

Abbreviations: To, annealing temperature; UPL, Universal Probes Library (Roche, Germany).

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Cγ ameliorates pancreatic GLUT2 protein over-expression to similar levels found in healthy animals and increases Pdx-1 and Slc2a2 gene expression in pancreatic tissue in diabetic rats.

We used immunohistochemistry to evaluate GLUT2 protein content in pancreatic islets. A semi-quantitative analysis was performed to determine the GLUT2 positive area as percentage (%). Immunopositivity to GLUT2 in Langerhans islets of STZa rats was more than two-fold higher (21.64%) than in the Ctrl group (9.48%). Remarkably, Cγ administration to diabetes-induced rats reduced GLUT2 protein content (12.52%) to a level similar to that of the Ctrl group (Fig. 2A–C). The absence of cross-reactivity validated the immunohistochemistry assays (Fig. 2D).

Cγ-induced attenuation of GLUT2 protein expression in diabetic animals may be due to changes in circulating glucose levels. In this respect, previous data have shown an ability of Cγ to stimulate glucose uptake and activation of the insulin signaling cascade in vitro in C2C12 cells, which suggests a higher GLUT4 membrane translocation (Ferruzzi et al., 2011). However, further research is needed since other molecules, or signaling pathways may be involved.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>GLUT2 expression (% area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>9.48</td>
</tr>
<tr>
<td>STZa</td>
<td>21.64*</td>
</tr>
<tr>
<td>STZa-Cγ</td>
<td>12.52</td>
</tr>
</tbody>
</table>

Fig. 1. Effect of the daily intake (120 mg/kg BW) of lupin gamma conglutin (Cγ) on the glycemic status of streptozotocin-induced diabetic rats. Bars and figures represent the average serum glucose levels ± standard error of the mean, after seven days, for the control group without diabetes induction or treatment (Ctrl), the streptozotocin-induced diabetic group without Cγ treatment (STZa), and the streptozotocin-induced diabetic group treated with Cγ (STZa-Cγ). Horizontal lines across bars indicate the statistical significance of the difference between pre- and post-treatment glucose values (*p < 0.05).

Fig. 2. GLUT2 protein expression in Langerhans islets (40x) of experimental groups. Streptozotocin-induced diabetes results in a GLUT2 over-expression (B, STZa) as compared to the control group (A, Ctrl). Daily intake of gamma conglutin protein ameliorates GLUT2 over-expression (C, STZa-Cγ) in streptozotocin-induced diabetic animals. The negative control shows absence of cross-reactivity (D). GLUT2 positivity was expressed as immunoreactive area per total islet area (%). *p < 0.05, compared to Ctrl group. Abbreviation: GLUT2, glucose transporter 2.
Since GLUT2 immunopositivity was found distributed throughout the hepatic tissue, it was not possible to delimit the positive area to quantify the expression of GLUT2 protein, despite several technical modifications in the immunochemistry’s assay conditions.

On the other hand, expression of the genes Pdx-1 and Slc2a2 was higher in the pancreatic tissue of STZa-Cγ rats as compared to STZa group (Fig. 3). Pdx-1 is a master transcriptional regulator that modulates β-cell differentiation and function by stimulating the expression of Ins-1, Slc2a2, and Gck genes among others (Ohlsson et al., 1993; Watada et al., 1996; Waeber et al., 1996). A reduced Pdx-1 gene expression leads to hyperglycemia through loss of β-cell function, which is related to down-regulation of Slc2a2 and Gck (Ahlgren et al., 1998; Gao et al., 2014). Slc2a2 and Gck play a prominent role in glucose-stimulated insulin secretion (GSIS) by pancreatic β-cells. Therefore, a decreased Slc2a2 expression is associated with β-cells’ unresponsiveness to hyperglycemia (Unger, 1991; Thorens et al., 1992; Guillam et al., 1997; Weir and Bonner-Weir, 2004). Our results show a lack of positive correlation between mRNA (Slc2a2) and protein (GLUT2) levels. We observed a decreased GLUT2 content and a slightly increased Slc2a2 mRNA levels in pancreatic tissue after Cγ treatment; however, this effect has been reported for other compounds and explained through protein’s half-life modulation (Gremlich et al., 1997).

Unfortunately, it was not possible to quantify expression of the Gck gene by qPCR in pancreatic tissue, possibly due to an elevated rate of β-cells death after STZ administration.

**Slc2a2 and Gck mRNA levels in hepatic tissue of Cγ-treated rats**

We also measured the expression of the Gck and Slc2a2 genes in hepatic tissue of experimental animals (Fig. 4A and B). As compared to the control group, both STZa and STZa-Cγ showed a significant increase in Slc2a2 gene expression (Fig. 4A) \( p < 0.05 \) and agree with previous reports in diabetes-induced rats (Yonamine et al., 2016). However, the Cγ-treated group showed even higher Slc2a2 gene expression. The increase in hepatic Slc2a2 gene expression after Cγ treatment might be indicative of augmented glucose uptake, as observed in hepatic cells in vitro (Lovati et al., 2012). This mechanism might explain the reduction of circulating glucose in STZa-Cγ rats (Lovati et al., 2012).

Although Gck levels were expected to increase after Cγ treatment, we found decreased Gck gene expression in both, STZa and STZa-Cγ groups, as compared to the Ctrl rats \( p < 0.05 \) (Fig. 4B). It is possible that Cγ had a transient effect on Gck mRNA levels (Arden et al., 2011). Studies with GCK activating molecules have shown this type of response (Agius, 2014).

On the other hand, recent reports indicate that the steroid receptor coactivators (SRC) participate in the transcriptional regulation of both, G6pc and Gck genes. The loss of Src2 causes down-regulation of G6pc and Gck gene expression in the liver of Src2 knockout mice (Fleet et al., 2015). In this respect, the regulation of Gck by SRC might be responsible for the low levels observed in this study. Moreover,
this might explain the Gck and G6pc down-regulation after Cy treatment (Gonzalez-Santiago et al., 2017). Whether or not Cy affects the expression of this group of coactivators remains to be determined.

Altogether, these data indicate a higher hepatic glucose uptake, but whether the glycolysis rate is impacted or alternative glucose metabolism routes are activated is not known and needs further investigation.

Glycogen content in hepatic tissue of Cy-treated rats

To further explore the metabolic fate of internalized glucose in the hepatic tissue of diabetes-induced rats treated with Cy, we performed a periodic acid-Schiff staining (PAS) to reveal glycogen deposits. As expected, after an overnight fasting period, glycogen reservoir was depleted in Ctrl rats (0% (Fig. 5A). On the contrary, STZa animals showed a highly preserved glycogen content after this non-feeding glycogen content (45% of positivity to the stain) (Fig. 5B). Similar to the Ctrl rats, STZa-Cy had lower glycogen content as shown by a decreased PAS positivity after a 7-consecutive-day Cy treatment (22.5%) (Fig. 5C). In addition, the glycogen distribution was markedly different between hepatocytes from STZa and STZa-Cy rats. Whereas the glycogen was observed homogenously and completely distributed in the cytoplasm of hepatic cells from STZa animals, glycogen deposits were found focalized inside the hepatocytes of STZa-Cy rats (Fig. 5A–C).

Accordingly, other authors have reported a significant increase in hepatic glycogen content of animals induced to diabetes with STZ after a 16-h fasting period as compared to non-induced control rats (Ugochukwu and Babady, 2003). Other authors have found that after STZ-induced diabetes, a significant reduction in glycogen phosphorylase is observed, at both mRNA and enzymatic activity levels (Rao et al., 1995). Therefore, our results might be a consequence of the diabetes induced by STZ.

On the other hand, ~80% of total gluconeogenesis is attributed to hepatic glucose production. Additionally, it has been shown that hepatic gluconeogenesis plays a major role in hyperglycemia during the fasting and the feeding states (Sharabi et al., 2015). Thus, an augmented gluconeogenic rate in hepatic tissue is observed in the diabetic state (Lin and Accili, 2011). The increase in hepatic glycogen synthesis in STZa animals without treatment correlates with higher Gck mRNA levels, possibly indicating a higher rate of glucose phosphorylation. Nonetheless, G6P can be directed either to glycolysis or glycogen synthesis. Altogether, our data suggest a modulation of glycogen metabolism in STZa-Cy rats, resembling the findings in the Ctrl group.

The PAS staining has the disadvantage of being unspecific for glycogen detection. Therefore, we performed a diastase-PAS staining to corroborate the presence of glycogen in the histological sections. After a diastase-digestion, we confirmed that the PAS-positive sections corresponded to glycogen (data not shown).


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