Pancreatic response of rats fed genetically modified soybean

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ABSTRACT: Mice fed genetically modified (GM) soybean were not affected in nutritional performance, but pancreatic microscopic features were disturbed. The mechanisms for these contradictory findings are unknown. This study analysed the histology of acinar pancreatic cells and the expression of pancreatitis-associated protein (PAP) and trypsinogen mRNA in rats fed GM soy protein. Two bioassays were run, each one with 34 Wistar rats distributed into two groups fed with non-GM or GM-soy protein (18% protein) for 0, 1, 3, 5, 15 and 30 days. Nutritional evaluation, plasma amylase levels, pancreatic histochemical analysis and quantification of PAP and trypsinogen mRNAs levels using quantitative real-time RT-PCR were done. No differences in nutritional performance among rats fed non-GM and GM diets were found. The GM, but not the non-GM, diet induced zymogen-granule depletion after 15 days feeding, returning to normal levels after 30 days (P < 0.05). Acinar disorganization started as early as 5 days after initiation of the GM diet and it recovered after 30 days. Levels of PAP mRNA significantly increased in the GM diet between day 1 and day 3 and decreased to the basal level by day 15. Trypsinogen mRNA peaked at two different times; at day 1 and at day 15, decreasing to basal levels after 30 days. Plasma amylase levels remained unchanged at all times. This indicates that GM soy protein intake affected pancreas function, evidenced by the early acute PAP mRNA increased levels and pancreas cellular changes followed by recuperation of acinar cells after 30 days. Copyright © 2007 John Wiley & Sons, Ltd.

KEY WORDS: genetically modified soybean; pancreas; histological changes; trypsinogens and PAP mRNA; real-time PCR

Introduction

Genetically modified (GM) crops are currently exploited around the world for animal and human consumption. The main biotech crop is soybean, with 58.6 million hectares (57% of global biotech area) in 2006 (James, 2006). The herbicide tolerance of GM crops is one of its main traits, followed by insect resistance. Despite production benefits, the commercialization of biotech crops has raised concerns and protests from consumers, environmental groups, some governments and scientists. Proponents of agricultural biotechnology have suggested that this debate should be resolved by scientific research (Frewer et al., 2004).

There are few scientific papers stating the effects of GM foods on nutrition and health, contrasting with more than one thousand publications about its detection, new modifications, consumer acceptance, regulations, opinions about environmental risks and others (http://www.ncbi.nlm.nih.gov/pubmed). Additionally, the published nutritional evaluations are quite heterogeneous. GM-corn (Barriere et al., 2001; Brake et al., 2003; Brake and Vlachos, 1998; Donkin et al., 2003; Erickson et al., 2003; Hammond et al., 2004; Hammond et al., 2006; Ipharraguerre et al., 2003; Seralini et al., 2007; Yonemochi et al., 2002), potato (Hashimoto et al., 1999; Seek et al., 2005), tomato (Chen et al., 2003), soybean (Hammond et al., 1996) and other crops (Melander et al., 2003) have been evaluated for nutritional quality and gross aspect of organs and no adverse effects on health have been found. However, at the microscopic and ultramicroscopic levels, there were cellular changes attributable to GM food intake (Ewen and Pusztai, 1999; Fares and El-Sayed, 1998; Malatesta et al., 2003; Malatesta et al., 2002a, 2002b; Tudisco et al., 2006; Vecchio et al., 2004).

In this regard, it has been shown that mice chronically fed since gestation with GM soybean had problems in synthesis and processing of zymogens by pancreatic acinar cells, reduced nucleoplasmic and nucleolar splicing factors and perichromatin granule accumulation on...
pancreatic acinar cell nuclei (Malatesta et al., 2002b, 2003). Hepatocyte changes suggest high metabolic rate and intense molecular trafficking (Malatesta et al., 2002a). Lactic dehydrogenase (mainly LDH1 isoenzyme) was significantly increased in the kidneys and hearts of rabbits fed GM soybean (Tudisco et al., 2006). It appears that GM soybean affects animal physiology with no gross modifications of indicators such as total body and organ weights.

Some of the changes in pancreatic cells associated with GM-food consumption have also been observed in animal models of pancreatitis (Andrzejewska et al., 2005; Ding et al., 2003; Magaña-Gómez et al., 2006). Pancreatitis-associated protein (PAP) is highly induced in acute pancreatitis (Dusetti et al., 1995; Iovanna et al., 1991b; Magaña-Gómez et al., 2006). PAP mediates protection against pancreatic injury by suppression of local pancreatic and systemic inflammation during acute pancreatitis (Zhang et al., 2004). Trypsinogens are important constituents of zymogen granules and their expression is altered during pancreatitis (Iovanna et al., 1991b). This study hypothesized that the intake of GM soybean induces pancreatic stress or injury by analysing the cellular structure of acinar pancreatic cells and the expression of PAP and trypsinogens by qRT-PCR in rats fed GM soy protein for 30 days.

Materials and Methods

Materials

Two commercial soy protein isolates (SPI) labeled as GM (SUPRO 500E) or Non-GM (SUPRO 500E IP) were purchased and GM or non-GM origins were confirmed by PCR. Soybean powder containing 5% Roundup Ready™ soybean (Fluka Chemie, Geel, Belgium) was used as a positive control for DNA characterization and it was obtained from the Institute for Reference Materials and Measurements (Fluka Chemie, Geel, Belgium). Genomic DNA was isolated as previously described (Magaña-Gómez et al., 2003). Four different DNA fragments were detected by PCR: soybean lectin, an endogenous gene: CaMV promoter 35S (p35S), a specific transgenic sequence; 5-enol-pyruvylshikimate-3-phosphate synthase (CP4-EPSPS), responsible for herbicide resistance to glyphosate; and the NOS terminator (NOS-T) from Agrobacterium tumefaciens. All PCR reactions contained 1 U Taq DNA polymerase (Invitrogen Corp. Carlsbad, CA, USA), 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.8 mM MgCl₂, 200 μM each dNTP and 0.32 μM of forward and reverse primers (Table 2) in a total volume of 25 μl. Genomic DNA (200 ng) was used as a template. PCR conditions were 40 cycles of 30 s at 94 °C, 35 s at 63 °C, and 40 s at 72 °C, with 3 min at 94 °C prior to the reaction and 5 min of final extension at 72 °C in a MJ Mini Thermal Cycler (Bio-Rad, Hercules, CA, USA). PCR products were resolved in a 1% agarose gel prestained with ethidium bromide (0.1 μg ml⁻¹). Analyses of trypsin and chymotrypsin inhibitors (Oppert et al., 1997), genistein and daidzein isoflavones (Klump et al., 2001) and SDS-PAGE (Laemmli, 1970) of SPIs were done.

Experimental Design

Two independent bioassays of 34 male Wistar rats (Harlan Mexico) each were carried out. Bioassay 1 consisted of 4-week-old rats, mean weight 47.8 g and, bioassay 2 consisted of 5-week-old rats with a mean weight of 62.6 g. Rats were individually housed in metabolic cages (Nalgene). Bioassays were divided in two groups of 17 rats each. Each group was fed an isoenergetic diet of conventional (non-GM) or GM-SPI (18% protein). The diet composition is shown in Table 1. Animals were kept at 22–25 °C and a 12-h light:dark cycle, diet and water were provided

<table>
<thead>
<tr>
<th>Table 1. Composition of the experimental diets (g kg⁻¹)</th>
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<tbody>
<tr>
<td>Ingredient (g kg⁻¹)</td>
</tr>
<tr>
<td>SUPRO 500E IP Non-GM soy protein isolatec</td>
</tr>
<tr>
<td>SUPRO 500E GM soy protein isolatel</td>
</tr>
<tr>
<td>dL-Methionined</td>
</tr>
<tr>
<td>Minerals mixe</td>
</tr>
<tr>
<td>Vitamins mixe</td>
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<tr>
<td>Cellulosef</td>
</tr>
<tr>
<td>Corn oil</td>
</tr>
<tr>
<td>Choline chloridef</td>
</tr>
<tr>
<td>Sucrose</td>
</tr>
<tr>
<td>Cornstarch</td>
</tr>
</tbody>
</table>

a Non-GM: non-GM soy protein isolate diet.
b GM: GM-soy protein isolate diet.
c The Solae Company, St Louis, MO, USA.
d Dyets Inc., Bethlehem, PA, USA.
e AIN-76 mixture. Harlan Teklad, Madison, WI, USA.
f AIN-76A mixture. Harlan Teklad, Madison, WI, USA.

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DOI: 10.1002/jat
ad libitum. Rats were killed at 0, 1, 3, 5, 15 and 30 days after initiation of the bioassay. Previous to killing, the animals were starved for 9 h and, anesthetized with 10–15 mg of tiletamine chlorohydrate and zolazepam chlorohydrate (Zoletil 50\(^{®}\)) per kg of body weight. Blood samples were collected into polypropylene tubes containing EDTA, the plasma was separated by centrifugation for 20 min at 4 °C, and stored frozen at −70 °C until analysed. The pancreas was dissected and divided into two portions, for histological and gene expression analyses. The results of gene expression analysis were corroborated through a second bioassay in similar conditions to the first bioassay. The feeding protocol and animal handling were approved by the local ethics committee.

**Diet Performance**

Nutritional quality of the diets was evaluated by ingested food, feed conversion (FC) and protein efficiency (PE). FC was determined by the weight gained (g)/food intake (g) ratio. PE was obtained by the weight gain (g) divided by protein intake. Both indicators (FC and PE) were calculated from the estimated values of each dietary sub-group at day 30 of the experimental period. Food consumption registries were taken every 3 days.

**Plasma Amylase Levels**

Plasma amylase was determined using a commercial kit (Randox Laboratories Ltd, Crumlin, UK) following manufacturer’s instructions.

**Histological Examination**

Pancreas sections were fixed in 10% formaldehyde, embedded in paraffin and 3–5 μm thick sections were cut with a semi-motorized rotary microtome (Leica RM-2145) and mounted on slides. Sections were stained using hematoxylin and eosin (H & E) procedure and examined by objective and feeding period were recorded by a microscope video camera. Tissues were blind-scored by a pathologist, according to Kyogoku _et al._ (1992). Images were scored for zymogen granule depletion (0, absent; 1, <20%; 2, 20–50%; 3, >50%); interstitial edema (0, absent; 1, expanded interlobular septa; 2, expanded intralobular septa; 3, separated individual acini); polymorphonuclear neutrophil infiltration (PMNi) [0, absent; 1, <20 PMNs per intermediate power field (IPF); 2, 20–50 PMNs per IPF and 3, >50 PMNs per IPF] and vacuolization, based on the percentage of acinar cells with cytoplasmic vacuoles per IPF (0, absent; 1, <20%; 2, 20–50%; 3, >50%) and acinar disorganization based on the percentage of area with abnormal acinar distribution (0, absent; 1, <20%; 2, 20–50%; 3, >50%).

**Statistical Analysis**

Protease inhibitors and isoflavones, diet performance, plasma amylase and histological examination were expressed as mean ± standard error of the mean (SEM). Statistical analyses of the mean values were done using the general linear models procedure, followed by comparisons using the Tukey-Kramer multiple comparisons procedure. _P_ < 0.05 was considered as the significance limit. The statistical program NCSS 2001 was used (NCSS, 2001) for all the analyses.

**RNA Preparation and Reverse Transcription**

Dissected pancreases were immediately rinsed with RNase-free water, homogenized in 1 ml of TRIZol\(^{®}\) Reagent (Invitrogen, Carlsbad, CA, USA), frozen and stored at −70 °C. Prior to reverse transcription (RT), potentially contaminating residual genomic DNA was eliminated with DNase I. The RT reaction was done in a total volume of 21 μl containing 2 μg of total RNA, 1 μl of 0.5 mg ml\(^{-1}\) oligo(dT)\(_{15}\), 40 U of RNaseOUT, 2.5 mM dNTP, 200 U of M-MLV RT and 5× RT Buffer. All components were purchased from Invitrogen (Carlsbad, CA, USA). Synthesized complementary DNA (cDNA) was stored at −20 °C until use.

**Quantitative PCR Analysis**

Real-time RT-PCR quantification (qRT-PCR) of PAP-, trypsinogen-, ribosomal protein L13A mRNAs and 18S rRNA was performed with an iCycler iQ detection system (Bio-Rad, Hercules, CA, USA). PAP mRNA (GenBank accession No. NM_053289.1) accumulation was used as an indicator of pancreatic injury as reported elsewhere (Magaña-Gómez _et al._, 2006). Trypsinogen mRNA (GenBank accession No. V01274 and V01273) was used to evaluate exocrine pancreas function. L13A mRNA (GenBank accession No. X68282) and 18S ribosomal RNA (rRNA) (GenBank accession number X01117) were also analysed to normalize data in bioassay 1 and 2, respectively (Jesnowski _et al._, 2002). Primers were designed using Primers3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_ www.cgi) and synthesized by Sigma Genosys (The Woodlands, TX, USA); primer sequences and PCR product sizes are listed in Table 2. Gene quantification was done using SYBR Green I. For 25 μl of reaction, 1 μl of cDNA (corresponding to 100 ng of the original total.
Table 2. Primers used for PCR and their characteristics

<table>
<thead>
<tr>
<th>Primer name (Fw/Rv)</th>
<th>Nucleotide sequence (5'-3')</th>
<th>Specificity</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL for</td>
<td>ATGGGGCTGCTTCTTCTTCT</td>
<td>Soybean lectin</td>
<td>157</td>
<td>Germini et al., 2004</td>
</tr>
<tr>
<td>SL rev</td>
<td>CCGATGTTGAGATTTGGTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM01</td>
<td>CACTAATAATGCGATCTATCCGATA</td>
<td>CaMVp35S</td>
<td>218</td>
<td>Quist and Chapela, 2001</td>
</tr>
<tr>
<td>CM02</td>
<td>CTTATATAGGAAGGCTTTGGGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RR04</td>
<td>CCCCCAGTCCTCAATTCCTCAAGT</td>
<td>CP4-EPSPS</td>
<td>180</td>
<td>Studer et al., 1998</td>
</tr>
<tr>
<td>RR05</td>
<td>GCCTGAGGGCCGCTCTGCTGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nosterfor</td>
<td>GAATCTCTGTGCGGCTTGGG</td>
<td>NOS-terminator</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>Nosterrev</td>
<td>GCGGGACTCTAATCATAAAAACCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAPfw</td>
<td>TGAAATTATGCAACCTGGAGAGG</td>
<td>PAP</td>
<td>318</td>
<td>Magaña-Gómez et al., 2006</td>
</tr>
<tr>
<td>PAPrv</td>
<td>TTACTGCTTTCCAAGACATGAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rTRY3/Fw</td>
<td>TYTTGGAGGYTCCCCTCATC</td>
<td>Trypsinogens</td>
<td>226</td>
<td></td>
</tr>
<tr>
<td>rTRY3/Rv</td>
<td>CORMATTGAGKKTSCAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rL13A/fw</td>
<td>AAGCAGGATCTGGCTGGG</td>
<td>L13A ribosomal protein</td>
<td>261</td>
<td>Magaña-Gómez et al., 2006</td>
</tr>
<tr>
<td>rL13A/rv</td>
<td>CCAACACCTTGGAGGCTTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r18S/Fw</td>
<td>GCAATTATTCGCCATGAAGG</td>
<td>18S rRNA</td>
<td>187</td>
<td></td>
</tr>
<tr>
<td>r18S/Rv</td>
<td>AGTTCACTGCTCTTCTTCAAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Y, C + T; R, A + G; M, A + C; K, G + T; S, G + C.

RNA, 0.9 μL forward primer (10 μM), 0.9 μL reverse primer (10 μM), 12.5 μL SYBR Green PCR Master Mix (PE Applied Biosystems) and 9.7 μL PCR-grade water, were mixed. PCR parameters for quantifying PAP mRNA levels were 94 °C for 4 min, 1 cycle, then 94 °C for 30 s, 61 °C for 30 s and 70 °C for 30 s, 40 cycles. PCR parameters for trypsinogen mRNA quantification were 94 °C for 6 min, 1 cycle, then 94 °C for 35 s, 58 °C for 35 s and 72 °C for 40 s, 40 cycles. PCR parameters for L13A quantification were 94 °C for 4 min, 1 cycle, then 94 °C for 30 s, 68 °C for 35 s and 70 °C for 40 s, 6 cycles, then –0.3 °C from 68 °C for 35 s and 70 °C for 40 s, 34 cycles. PCR parameters for 18S rRNA quantification were 85 °C for 30 s, 94 °C for 6 min, 1 cycle, then 94 °C for 35 s, 60 °C for 35 s and 72 °C for 40 s, 40 cycles. In all cases, a single fluorescence measurement was made at the end of each extension segment. A melting curve program (60–94.5 °C with a heating rate of 0.3 °C, 20 s with continuous fluorescence measurement) and a cooling step to 4 °C, were added.

The relative abundance of mRNA in each pancreatic section was measured according to the Pfaffl method (Pfaffl, 2001), using the formula

\[
\text{Ratio of gene expression} = \left( \frac{E_{\text{target}}}{E_{\text{ref}}} \right)^{\Delta C_i} = \frac{E_{\text{target}}}{E_{\text{ref}}}^{\Delta C_i}, \text{ target} (\text{control} - \text{sample}) \]

The above algorithm was used to calculate the results from amplification, where \(E_{\text{target}}\) and \(E_{\text{ref}}\) are the efficiencies of the target (PAP and trypsinogens) and reference genes (L13A and 18S) respectively and \(\Delta C_i\) is the difference between the mean \(C_i\) of control cDNA (non-GM diet) and sample cDNA (GM diet). Results were analysed by the absolute fluorescence increase method using the LinRegPCR software v.7.5. Briefly, this method measures the actual efficiency of each amplification curve by fitting its linear part in a simulation plot of the Log (fluorescence) versus Cycle in the exponential phase of amplification and calculates the efficiency from the slope of a linear regression model of the simulation curve (Pfaffl, 2001).

All the amplifications had PCR efficiencies from 1.9 to 2.0 and correlation coefficients above 0.999. The ratios of expression values for GM relative to non-GM diet for each period were plotted in Excel (Microsoft, Redmond WA).

Results

Sample Characterization

Specific PCR products were obtained for DNA characterization (Fig. 1). As expected, the soybean lectin gene (157 bp) was detected in all the samples except for the no-template control (NTC). Fragments of 180, 218 and 125 bp (CP4-EPSPS, CaMVp35S and T-NOS, respectively) were obtained in the SUPRO 500E sample and the positive control, but not in the SUPRO 500E IP and NTC. Therefore, SPI SUPRO 500E IP was non-GM and SUPRO 500E was prepared from glyphosate tolerant soybean (GTS) event 40-3-2.

Both SPI samples were analysed by SDS-PAGE and similar electrophoretic protein patterns in both GM and non-GM protein isolates were obtained (data not shown). Trypsin inhibitor activity (4.84 vs 4.36 mg trypsin inhibitor g−1 protein for GM or non-GM SPI, respectively) and chymotrypsin inhibitor activity (0.71 vs 0.69 mg g−1 protein for GM or non-GM SPI, respectively) were similar between samples. Isoflavones genistein and daidzein were higher in non-GM SPI (52.6 vs 25.9 and 92.9 vs 49.2 μg g−1 sample, respectively) than in GM SPI.
PANCREAS RESPONSE TO GENETICALLY MODIFIED SOY

Figure 1. Qualitative PCR analysis of soybean lectin, CP4-EPSPS, CaMV 35S and NOS terminator to characterize soybean samples. M, PCR marker 100 bp DNA ladder; NTC, no template control; + Ctrl, GM reference material; SPI 500E, soybean protein isolate 500E; SPI 500E IP, soybean protein isolate labeled as Non-GM. Lane 1, soybean lectin (157 bp); lane 2, CP4-EPSPS (180 bp); lane 3, CaMV p35S (218 bp); lane 4, NOS terminator (125 bp)

Table 3. Growth and nutritional performance of rats fed GM or non-GM diets

<table>
<thead>
<tr>
<th></th>
<th>Bioassay 1</th>
<th></th>
<th>Bioassay 2</th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>GM diet</td>
<td>Non-GM diet</td>
<td>GM diet</td>
<td>Non-GM diet</td>
</tr>
<tr>
<td>Ingested food (g) day 3</td>
<td>26.3 ± 0.92</td>
<td>28.9 ± 0.92</td>
<td>36.3 ± 1.26</td>
<td>34.9 ± 1.26</td>
</tr>
<tr>
<td>5</td>
<td>56.9 ± 2.14</td>
<td>57.9 ± 2.14</td>
<td>57.2 ± 1.12</td>
<td>60.9 ± 1.12</td>
</tr>
<tr>
<td>15</td>
<td>201.2 ± 7.87</td>
<td>209.1 ± 7.87</td>
<td>197.2 ± 0.65</td>
<td>197.6 ± 0.65</td>
</tr>
<tr>
<td>30</td>
<td>536.8 ± 13.8</td>
<td>524.2 ± 13.8</td>
<td>509.1 ± 2.9</td>
<td>507.4 ± 2.9</td>
</tr>
<tr>
<td>Weight gain (g) day 3</td>
<td>17.4 ± 0.59</td>
<td>16.5 ± 0.59</td>
<td>21.4 ± 1.21</td>
<td>21.9 ± 1.21</td>
</tr>
<tr>
<td>5</td>
<td>27.1 ± 1.04</td>
<td>29.3 ± 1.04</td>
<td>37.3 ± 1.60</td>
<td>41.0 ± 1.60</td>
</tr>
<tr>
<td>15</td>
<td>93.6 ± 3.41</td>
<td>93.9 ± 3.41</td>
<td>88.3 ± 1.49</td>
<td>91.5 ± 1.49</td>
</tr>
<tr>
<td>30</td>
<td>192.6 ± 8.39</td>
<td>190.1 ± 8.39</td>
<td>191.7 ± 5.52</td>
<td>197.6 ± 5.52</td>
</tr>
<tr>
<td>Feed conversion</td>
<td>0.36 ± 0.008</td>
<td>0.36 ± 0.008</td>
<td>0.38 ± 0.01</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>Protein efficiency</td>
<td>1.85 ± 0.04</td>
<td>1.85 ± 0.04</td>
<td>1.94 ± 0.05</td>
<td>1.99 ± 0.05</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. Analysis was made for each bioassay. a Calculated from the group surviving at day 30. All values were not statistically different, P > 0.05.

Diet Performance

The nutritional quality of the diets was similar for the non-GM and GM group in both bioassays. No differences were found in the amount of ingested food (P > 0.05) (Table 3). Body weight gain was similar among groups (P > 0.05). Likewise, food conversion and protein efficiency values were comparable (P > 0.05) between the non-GM and GM diets during the study and after 30 days feeding (Table 3).

Plasma Amylase

Amylase levels measured in bioassay 1 were increased starting on day 3 for both diets. However, lower levels were found from day 5, in rats fed the GM diet with respect to the non-GM group (Fig. 2).

Histological Examination

Acinar cells showed normal pancreas architecture at the beginning (0 h) of the experiment (data not shown). The basal portion of the cells contained the nucleus and the apical part, the secretory vesicles and the zymogen granules. Pancreas did not show evidence of edema, PMNi or vacuolization along the bioassay. Yet the GM diet induced zymogen granule depletion starting at day 5 and until day 15; this was significantly higher compared with the non-GM diet (P < 0.05). The zymogen granule content was fully recovered by day 30 (Fig. 3A). Similarly, acinar disorganization was suddenly increased in pancreas of rats fed the GM diet on day 5, remained high until day
Figure 3. Time course of histological changes in pancreas of rats fed on non-GM or GM diet. Zymogen granule depletion (A) and acinar disorganization (B). Each point represents the mean ± SEM. Asterisks show differences between diets, $P < 0.05$

15, and recovered thereafter (Fig. 3B). Microphotography of the characteristic histological changes of pancreatic acinar cells is shown in Fig. 4.

Quantification of PAP and Trypsinogen mRNA

Quantitative RT-PCR analysis was used to evaluate the abundance of PAP and trypsinogen mRNA in the pancreas of non-GM or GM fed rats. In both assays, PAP transcripts were detectable starting at day 1 in the GM group. Maximal accumulation was detected after 3 days or 1 day feeding in bioassay 1 and 2 respectively, with respect to the non-GM group (Fig. 5 A, B). After 5 days, PAP mRNA abundance was completely attenuated in bioassay 1, while in bioassay 2 the relative values decreased (GM:non-GM) by day 15 and remained similar in the rest of the experiment.

Changes in the relative accumulation of trypsinogen mRNA had a biphasic pattern. In both bioassays, trypsinogen mRNA index (GM:non-GM) was detected in days 1–3, high in bioassay 1 and modest in bioassay 2. Between days 4 and 5, the index was negative in bioassay 1, while it remained positive, although small, in bioassay 2. After 15 days feeding, trypsinogen mRNA increased several times in the GM with respect to the non-GM diet in both bioassays. On day 30, the GM/non-GM index of trypsinogen mRNA had a reduction of 1 in bioassay 1, although the values were above the control level in bioassay 2 (Fig. 6 A, B).

Discussion

Most GM plants contain the CaMV 35S promoter (Ahmed, 2002); for this reason, primers for this fragment were useful to screen for GM materials and the construct specific primers to classify the source of SUPRO 500E as glyphosate tolerant soybean (GTS 40-3-2). Components that could affect pancreatic function, such as trypsin and chymotrypsin inhibitors, were found on the normal range and in comparable levels between the GM and non-GM SPI (Anderson and Wolf, 1995). Lectin is considered an antinutrient in soybean; however, it is very labile and non detectable in soy protein isolates (Calderón de la Barca et al., 1991). Although the main soy isoflavones, daidzein and genistein, had a lower concentration in GM compared with non-GM SPI, these two isoflavones were found in the normal range for soy protein isolates (USDA, 2002; Wang and Murphy, 1994). Normal or low levels of isoflavones do not induce adverse effects (Constantinou et al., 2001). This study compared two commercial SPI, Non-GM and GM, identically processed by the same company and with a quite similar composition.

Animal growth and protein efficiency for both non-GM and GM soybean diets did not show differences, as reported previously (Hammond et al., 1996; Malatesta et al., 2002a; Tudisco et al., 2006). Even though the main biologically active factors were absent or comparable between the GM and non-GM soy isolates, significant histological and gene expression changes were detected in the pancreatic cells from rats fed on the GM diet with respect to those fed on the non-GM diet. The histological findings in rat pancreas as well as the effects on PAP and trypsinogen mRNA levels might suggest cellular injury attributable to GM soy protein consumption. Typical indicators of pancreatitis such as vacuolization or PMNi were not observed; however, acinar disorganization and zymogen granule depletion in the GM group were similar to those found in pancreatitis (Magaña-Gómez et al., 2006).

Changes of the zymogen area were comparable to the results of Malatesta et al. (2002b) for mice chronically fed on a GM soy diet, with an initial reduction of the total zymogen area of the pancreatic acinar cells and a later increase, resembling recuperation of the tissue. In our study, the process of differentiation and re-differentiation of pancreatic acinar cells explains the zymogen granule depletion and acinar disorganization as well as their recovery. Reconstitution of the normal morphology and
Figure 4. Representative light microscope sections of rat pancreas fed on non-GM or GM soybean diet. Sections show zymogen granule content (ZG, concentrated pink areas in the apical pole of the cells close to the acinar lumen) and acinar disorganization during the experimental time, according to diet. H & E staining (×132). Zymogen granule depletion (asterisk) and acinar disorganization (arrow) were different between diets on day 5 and day 15 ($P < 0.05$).

The increased abundance of PAP mRNA in the GM group with respect to the non-GM during the first days of feeding could be explained by the function of this protein. PAP was proposed as an acute phase protein expressed in defense reactions of pancreatic cells that induces suppression of local pancreatic functions, as well as systemic inflammation during acute pancreatitis or in subclinical pancreatic cell injury without pancreatitis (Savkovic et al., 2004; Paajanen and Nordback, 1999).

function of the pancreas involves cells proliferation from intact acini and tubular complexes (De Lisle and Logsdon, 1990; Lechene de la Porte et al., 1991). The changes observed in acinar disorganization and zymogen granules correspond to the transitional stages between acinar and ductular cells, where regular acinar cells are replete with dense zymogen granules, intermediate cells have a lower content of granules, de novo cells are empty and ductular cell lack them (Bockman, 1997).
Trypsinogen mRNA levels in both bioassays have patterns that are parallel to zymogen granule depletion. Trypsinogens are regulated at the transcriptional level (Iovanna et al., 1991a; Lhoste et al., 1994) thus, whereas accumulation occurs due to the absence of a stimulus, acinar cells appear empty of zymogen granules. Differences in the trypsinogen mRNA index (GM:non-GM) between bioassays are due to the use of different genes to normalize expression, since abundance of the ribosomal protein L13 mRNA is lower than 18S rRNA, used in each bioassay, respectively.

Amylase levels had a gradual increase during the bioassay due to the postnatal functional maturation (Githens, 1990). However, in agreement with the study of Malatesta et al. (2002b) in mice, a lower amylase content was found in pancreatic acinar cells in the GM-fed than in the non-GM-fed rats. Therefore, it is possible that the stimulated synthesis of trypsinogens related to the replenishment of the acinar cells exceeded the protein synthesis capacity of the exocrine pancreas, decreasing the synthesis of amylase.

The results appear to indicate that rats fed on a GM diet had a pancreatic supraphysiological stimuli or synergism with cholecystokinin (CCK); although not severe, it was sufficiently strong to induce a mild pancreatic injury with an adaptive response. Apparently, if endogenous CCK were over-expressed, a progressive deleterious effect must be induced, ending in pancreatitis and in this study, this situation was not present. This phenomenon could be explained from the results of a 30 day continuous pancreatic exocrine secretions model in rats (Miyasaka et al., 1992). In this model, duodenal mucosa CCK has three peaks on days 1, 3 and 14 and returns to basal levels on day 30. There were regenerative changes on day 7 and day 14, with almost a complete recovery of zymogen granules by day 14. In contrast to supramaximal CCK-8 or caerulein, acute or prolonged supraphysiological levels of endogenous CCK-58, the only detectable endocrine form in the rat (Reeve et al., 2003), do not cause pancreatitis (Yamamoto et al., 2007). The response of rat pancreatic cells is limited and a down-regulation of the receptor CCK-A has been suggested as a protective mechanism (Ohlsson et al., 2000).

Since the diet performance was similar between groups, it is possible that some bioactive components different to lectin or protease inhibitors could be affected by the genetic
modification itself and evoke a pancreatic response. Recently, it was demonstrated that transcriptional mRNA variants of the inserted construct occur on the GTS 40-3-2 (Rang et al., 2005). Therefore, not all the possible proteins expressed in the GM soybean have been evaluated, because the study to test toxicity of EPSPS was done using recombinant protein produced in bacterial cells (Harrison et al., 1996). In conclusion, our study shows mild damage with fast recovery of the pancreas in rats (30 days for rats) upon consumption of GM-based food, but humans or other animals with different response to continuous stimulus of endogenous CCK or under regime of intermittent feeding with GM soy protein, could be differently affected. Finally, the analyses of mRNA levels to evaluate the stressing effects caused by GM food are very sensitive protocols that can be used to evaluate this kind of effect in other mammals.

Acknowledgments—The excellent technical assistance provided by René Valenzuela and Sofía López Valenzuela is greatly appreciated. We are also grateful to Dr Amanda Galvez for her critical comments to the manuscript and to the Mexican Council for Science and Technology (CONACyT) for its financial support to this study (grant 43928 to AMC, and a Ph.D. scholarship to JAM).

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