BUDAPESTI CORVINUS EGYETEM

Élelmiszertudományi Kar

PhD thesis

STUDYING THE CONTAMINATION OF THE GLYPHOSATE- TOLERANT SOYBEAN FROM SOY CONTAINING FOOD PRODUCTS AND THE RISK OF ITS TRANSLOCATION INTO THE ORGANISM USING PCR METHOD

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Budapest

2009

PhD School/Program

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1. INTRODUCTION

Genetically Modified Organisms (GMO) are such living organisms in which the DNA has been modified in a way that cannot be found in nature. The first genetically modified plant has been created in 1984, and over 60 species has been genetically modified ever since. Most of our main food source plants are included in this 60 species either for research purposes or for agricultural use. Using genetic technology there is a possibility to cross non-related species' genes as well. We recognize first, second and third generation of GMO plants. The first generation is the biotic and abiotic stress resistant plants, most of which are the insect resistant and herbicide tolerant plants. Among these the most frequently seen ones are soybean, corn, rape and cotton. From the food industry's point of view the most significant is the soybean, especially for the meat industry, where it is used in large quantities as protein substitute or stabilizers. Second generation transgenic plants are modified in regards of growth metabolism, while the third generation is the so called bioreactor plants. The social acceptance towards GM plants is far from being unified, hence we must grant the freedom of choice for the consumers for growing and consuming GM plants. Taken all this into consideration the European Union controls by the means of regulations the producing, selling, tracking and marking of such crops and products. The 1829/2003 EU regulation concerns to the authorization, observing and labelling of the GM food and feed. According to the 1830/2003 EU regulation, any product containing more then 0.9% of GM component has to be labelled in all EU member states. The legal limit of GMO component or the total absence of such can be tested by certain analytical routines, most of which are suitable to test plant samples (seeds, livestock feed, and the raw and base materials of the food industry). To isolate DNA and to detect GMO from processed foods is usually very problematic, as certain food products' food-matrix contains proteins, fats, polysaccharides, polyphenols and other secondary components, which usually create an irreversible bond with the nucleic acids, furthermore the physical and chemical influence along the processing can also degrade the DNA. Because of the reasons mentioned above it is very important to further test adapt and improve the routines that we have for isolating DNA and to detect GMO.

In case of the gene technologically modified organism, containing or made of such organisms there are a number of environmental and ethical doubts, but more importantly, food safety issues are emerged as well, as the effect of eating GMO foods or foods containing GMO components, is not obviously clarified.

During the authorization process of a GM plant a number of risks estimated research has to be done. In case of GM foods (and feeds) the applicable research methods are elaborated by the biotechnological industry, the farmers, and the controlling authorities, taking mutual responsibility

along the process. Today, along the authorization process of a GM plant, the purpose of the modification has to be weighed, and also what new beneficial characteristics the new recombinant DNA containing plant will have. The GM crop line's new integrated risk assessment approach has to include: the parent crop's characteristics, the method of insertion of the donor and transgene, the description of the gene products, the food-safety characteristics of the new GM crop line, as well as the environmental risk assessment and the environmental effect monitoring and supervision.

Among the consumers the greatest worry was caused by the use of the virus promoters which play a role in the expression of the foreign gene. Based on the research of certain biotechnologists, we cannot rule out the possibility of the cauliflower mosaic virus promoter might activate inactive viruses, which might lead to growing new recombinant viruses, or as an other possibility, they might certain gene's increased operation. To see if this is really a threat that we need to take onto consideration we have to examine whether the cauliflower mosaic virus can penetrate into the bloodstream, organs or muscles.

2. GOALS

Taken what is mentioned above my goals were:

- Simple PCR examination of different GM soy containing meat products prepared industrial circumstances, coming from different technological phases, by this studying the DNA degradation caused by physical influence, and also whether this degradation effects the traceability,

- Adapting the DNA isolating methods recommended by the EU JRC (Joint Research Centre, Ispra), testing them, and improving them to be able to isolate DNA from different process stage of food matrixes. Elaborating and optimizing a cost effective three-phase partitioning based DNA extraction method and elaborating a DNA isolation technique for cocoa containing samples (chocolate and chocolate products) which are critical from the DNA extraction's point of view originated from commercial sources.

- Testing for the 0,9% labelling obligation set by EU regulations on commercial foods using qualitative and quantitative GM testing,

- Testing for the presence of the cauliflower mosaic virus 35S promoter from the Roundup Ready Soybean's expression vector in the nutrition pathway, and examining the penetration ability of 35S promoter into the organism by means of animal feeding tests by adapting and using the tests recommended by EU JRC.

3. MATERIALS AND METHODS

3.1. Examination Samples

3.1.1. Roundup Ready soy content detection in food samples

- The meat samples were produced by Hajdú BÉT RT in either Debrecen or Mezőkovácsháza. The samples contained turkey meat and they had 0.5%, 1%, 1.5% and 2% of Roundup Ready soybean flour mixed into them. The products that I have included in the examination, were poultry bolognas, ready-to-eat hamburgers, ready-to-eat hamburgers prepared by different processing techniques and turkey liver canes products taken from different technological phases.

- I have examined commercial samples as well, these were different meat products (for example bolognas, cold-cutlets, frankfurters, canned meats, liverwurst, canned livers and ready-to-eat products), baby foods (dairy powders and canned products), pizzas and pizza sauces, sweets (pudding powders, waffles, chocolates) and soy products (soy drinks, soy sauces, soy cubes, soy granulate, tofu).

3.1.2. Detecting the 35S promoter in the nutrition pathway and examining its penetration ability into the organism by means of rat feeding trial

I have used Wistar strain male rats which were kept on a soy free diet for 5 days prior to the experiment (5 specimens per group). During the experiment their food contained the total of 10% protein. I have mixed RR soy flour 0% (control group), 0.9%, 2.5% and 50% respectively into their food for 14 days. After over anesthetizing the rats, I have removed the spleen, liver, pancreas, kidneys, mesenteric lymph node, intestines, stomach, and muscle tissue, and also intestinal and stomach fluids.

3.1.3. Detecting the 35S promoter in the nutrition pathway, and examining its penetration ability into the organism by means of chicken feeding trial

The feeding experiment took place at the poultry processing plant of Master-M Ltd. in Kisvárda using 0% and 2,5% RR soy content feeds during the 42 days feeding period, and we took samples randomly 5 times. During every sampling I examined 3 specimens of which I have removed the gizzard, the crop, intestines, pancreas, mesenteric lymph node and the liver, and also took muscle tissue samples, thigh and breast as well as intestinal fluid.

3.2. Examination methods

For the GMO research I have used the EU JRC (Ispra) recommended method.
For the meat sample examinations I have used Wizard, Wizard combined with Amicon ultra filter, CTAB combined with proteinase-K enzyme, three-phase partitioning and DEAE cellulose ion changed chromatography methods.
Wizard method and Wizard method combined with Amicon ultra filter were used for isolating DNA in case of both feeding trials. The quality and quantity of the DNA solution (R value) were determined by spectrophotometric analysis based on the absorption ration measured at 260 nm and 280 nm.

During my research simple and real-time PCR system were used. For both the model meat samples and the commercial samples the absence of the DNA inhibitor and the presence of the soy were tested by amplification of the soy specific lectin gene. For the samples with no soy content the absence of inhibitors was tested using plant specific or vertebrate specific primers. In case of the animal feeding trial the absence of the inhibitors was tested using vertebrate specific primer pairs.

In my case the GMO detection is based on the detection of the regulator elements, meaning that in both the model and commercial samples. I have tested for the presence of the S35 promoter and/or *nos* terminator. During the tests the samples were run in 10% polyacrylicamid gel, stained with SYBR green I. for 15 minutes and documented using a Kodak EDAS 290 system.

Along the commercial sample tests, if the samples tested positive for the S35 promoter and/or the *nos* terminator, I have determined the GMO content by real-time PCR (GeneAmp 5700). Roundup Ready event specific primers and probes (TaqMan) and RR soy standard reference materials were used for the quantification (Fluka, ERM-BF410 - 0%, 0.1%; 0.5%; 1%; 2%, 5%).

4. RESULTS

4.1. Testing food samples to detect GMO

Based on the results of the meat samples' DNA isolation we can conclude that in every case the applied Wizard method yielded sufficient amount and purity DNA solutions, which were suitable for the further PCR tests. The lectin gene was detected in every sample, which means that the isolated DNA solutions were inhibitor free. Summarizing the model meat sample test results of the GMO specific PCR, we can conclude that with the adapted simplified PCR method, the polyacrylamide gel based separation and staining with SYBR green I. gave the required 0.5% detecting limit even in the case of the sterilized canned turkey liver products. By this we can easily fulfil the EU directive's 0.9% labelling obligation.

In case of the commercial samples the DNA isolation was performed with the Wizard method and the Wizard method modified with Amicon ultra filter and CTAB methods. I have developed a cost effective three-phase partitioning based DNA separating method for the different process stage model and commercial meat products, and I also developed an ion changed chromatography method for extracting DNA for commercial cocoa containing samples. Summarizing the commercial samples DNA isolating results, it is clear that 85 out of the 91 samples gave sufficient amount and quality DNA for the polymerase chain reaction (I have had no information on 5 ham product's soy content because these were not packed but freshly sliced products, so I excluded these from the further evaluation). I have experienced problems with the high processing level and carbohydrate containing pudding powders and soy sauces.

Summarizing the commercial food samples' lectin PCR results, out of 80 good quality DNA samples 65 was tested positive for lectin gene. 11% of the products were tested positive for the lectin gene in spite of the fact that the labelling had not indicated such, so those labelling were defective.

When detecting the GMO in the commercial samples 29 out of the 65 positive samples were tested positive for S35 promoter and/or *nos* terminator as well. None of the product labels had any indication of GMO content. Based on these result I can conclude that 47% of the tested samples gave a positive result for GMO presence. According to the quantitative results 12 out of the 29 positive samples had lower than 0.1% RR soy content (41.4%), 12 samples was in the range of 0.1% -0.9% (41.4%), and 5 samples (17.2%) was above the 0.9% RR soy content.

4.2. Detecting the 35S promoter in the nutrition pathway and examining its penetration ability into the organism by means of animal feeding trial

Along both feeding testes the applied methods yielded good quality and quantity DNA from the certain organs and muscle tissue, and the absence of the inhibitor was proven as well.
The rat experiment's results showed that in case of the samples coming from the group that has been fed with the 50% RR soy content feed some samples from stomach, intestines and fluids were tested positive for the 35S promoter. The explanation is that there was 12 hour of food absence before the anaesthesia, and this time is probably not long enough for the food to completely clear from the body. The examined organs and muscle tissue were negative every time meaning that neither the 35S promoter nor its smaller fragment was able to get through the intestinal wall hence not getting into the bloodstream. In case of the 2.5% RR soy content feed I only found 1 positive test from a stomach liquid sample. The rest of the specimens, including the group with the 0.9% RR soy content feed were completely negative for everything I have tested.

The chicken feeding experiment showed that the specimens taken from certain growth phases occasionally showed positive results for the samples that came in direct content with the feed (crop, inside of the gizzard and intestines) but the most commonly eaten giblets like the liver, or the muscle tissue samples always produced negative results. The samples taken from the processing line every single sample gave a negative result, which can be explained by the fact that before the processing the chicken undergoes an 8-12 hour starvation. During this time the specimen could digest the feed and it could clear from the body completely. Probably the reason why in case of the chicken feeding trial was not possible to detect anything from those samples, derived from the processing line, is that the digestive system of the chickens are different from the digestive system of the rats.

5. CONCLUSIONS AND RECOMMENDATIONS

For certain commercial samples that are critical from the DNA isolation's point of view (liquids, low DNA content, cocoa content or highly processed products) the DNA isolating method to detect GMO recommended by JRC has not yielded sufficient amount and quality DNA. To ensure to successful examination of such critical samples, the DNA isolating techniques need to be improved. The DEAE cellulose ion changed chromatography based DNA isolating method can be suitable for high carbohydrate or cocoa containing (chocolate, chocolate contained waffles, pudding powders) samples to obtain good quality amplifiable DNA. In case of samples with low DNA concentration,

the JRC recommended DNA isolating system is suitable, combined with Amicon ultra filter. The new low cost three-phase partitioning DNA extraction method could be used not only for GMO detection, but other PCR tests as well (meat origin, plant origin). I think the TPP method would be fine to test with real-time PCR comparing the most frequently used Wizard method's DNA solution's real-time PCR results.

In the literature there are only few GMO detection experiments documented, especially regarding sterilized meat products. For the future, I recommend meat products and foods with known RR soy content to be tested with real-time PCR method, both the raw materials and theirs processed products. This research would show how the different levels of heat treatment or other physical or chemical treatment effects the GMO content. For example would we see the same GMO content level in the processed liver canned as we see in the raw liver mass?

In case of the commercial samples the quantitative PCR based GM tests show that 17% of the GM positive products were exceeding the 0.9% labelling limit. Since none of these products' labels had any indication of the 0.9% exceeding GMO level, I would recommend to raise number of the yearly tested samples, to protect the customers.

I have tested the 35S promoter for detection in the nutrition pathway, and examining its penetration ability into the organism by means of animal feeding trial (rat and poultry feeding tests) using the DNA isolating and GMO detection methods recommended by JRC.

According to the short term rat feeding experiment, the 123 bp size fragment of the S35 promoter could not be detected in any organ samples, meaning that it did not get through the intestinal wall, and did not infiltrate the bloodstream either. In some cases I have got a positive signal for the 35S promoter, but only in samples from organs that came in direct contact with the food (stomach, intestines) and also in the stomach and intestinal liquids as well. The explanation for the positivity is probably that the starvation before the anaesthesia of the rats was not enough for the food to completely pass from the body. A more detailed research on this topic is necessary, with more animals, and more repetitions to determine the consumption risk.

According to the chicken feeding tests' results, in samples from the different feeding phases the ones that came in direct contact with the food (crop, inside of the gizzard and intestines for example) the S35 promoter was detected. The most frequently consumed parts like liver or muscle tissue were tested negative every time. This first result is positive regarding the GM feed consuming domestic animals, but I recommend further tests to strengthen this finding.

In the future I recommend carefully planned long term multi-generation animal tests, to determine whether consuming GM modified plants means any short term or long term risks or not, especially and directly in case of the plants that are processed to food.

6. NEW SCIENTIFIC RESULTS

1. I have adapted the DNA based PCR method to detect GMO recommended by the EU JRC (Ispra) and tested on different processing level of RR soy containing meat products and on food-matrixes of commercial origin. I have proven that as a result of heat treatment the detection ability of the *nos* terminator is reduced. Using the adapted and optimized simple PCR method the 0.5% detection limit could have been achieved even in case of the sterilized products.

2. I have combined the DNA isolating method recommended by the EU JRC with Amicon Ultra filter, which improved method proved to be suitable for DNA isolation from even diluted, low DNA level samples. I developed a new three-phase partitioning based method which is not only suitable for detecting GMO, but also can be used with other PCR examinations. For the cocoa containing samples I developed a DEAE cellulose based ion exchanged chromatography DNA extraction method, because in case of such materials the regularly and widely used methods are not sufficient enough.

3. I am one of the first scientists in Hungary to detect the RR soy on the almost complete range of foods. Based on the results I have concluded that 47% of the commercial samples contained GMO, and 17% of the GM positive products exceeded the legally set 0.9% labelling obligation. None of the 0.9% exceeding samples had any kind of indication on the label for GMO content.

4. I am the first scientist researching the possibility of detecting the cauliflower virus 35S promoter, which is in the expression vector of the Roundup Ready Soy, in the nutrition pathway, and examining the penetration ability of 35S promoter into the organism by means of rat and chicken feeding tests. I have adapted the DNA isolating and GMO detection methods recommended by the EU JRC for these experiments. Concluding these activities the 35S promoter was not detectable in the tested organ and muscle samples, meaning that the promoter could not go through the intestinal wall, nor could get into the blood stream.

PUBLICATIONS CONNECTED TO THE TOPIC

I. Articles in professional journal

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