PhD thesis

DEVELOPING MOLECULAR BIOLOGICAL METHODS FOR GLUTEN-FREE CONTROLLING

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1. INTRODUCTION

Coeliac disease (gluten-sensitive enteropathy) is an autoimmune disease triggered by consumption of cereal (wheat, rye, barley, triticale, oat(?)) prolamins, like gluten. The substance is, that these prolamins can cause the characteristic symptoms of coeliac disease, like damaging the small intestine in subjects who are predisposed to it. Therapy consists of a gluten-free diet. Although nowadays oats are generally considered not to be harmful, it has to be pointed out that it is still a potential problem that oats are frequently contaminated by wheat, therefore it is also prohibited in the coeliac diet. There are no conclusive clinical data on the threshold of gluten sensitivity of coeliac patients. Contamination of foodstuff constituents and inadvertent dietary transgressions are not rare. Accordingly, the food producers have to guarantee that their products are free from gluten, otherwise the labelling of gluten content is obligatory.

For this purpose considerable progress has been made in gluten analysis of food, immunochemical assays (e.g. ELISA) and non-immune methods have been introduced. Sensitivity, specificity and reproducibility of earlier protein based methods for gluten analysis were unsatisfactory, since the protein conformation usually changes dramatically during the technological process applied in the food industry. DNA-based PCR method provides new facilities to detect gluten contamination in processed foodstuffs. The aim of the application of PCR method is not to substitute ELISA, but to find a good complementary opportunity to detect gluten, or other toxic prolamin contamination. Furthermore, this could be a possibility to prove the presence of some „hidden" allergens, like wheat starch in foodstuffs originally free from allergen residues.

The present study describes the evaluation of polymerase chain reaction systems as possible indicators of contamination of gluten-free food with the celiac-toxic cereals.

1.1. Aims

The objective of my PhD thesis can be determined as:

- To adapt primer pair combinations in order to be able to detect dependably the presence of different species of wheat and other cross-reacting cereals growing in Hungary from large spectrum of foodstuffs.

- To be able to prove the amplicability (A49855/B49317 primer pair), the origin (TR01/TR02 primer pair; WBR11/WBR13 primer pair) of the plant DNA samples izolated from different cereals and of the DNA samples izolated from technologically processed foodstuffs. Further goal was also to allocate the presence of the allergen coding DNA sequence in mentioned samples.

- To test these adapted and developed methods on determined critical points of a gluten-free product manufacturing technology, and this way the contamination risk could be easily determined. After an artificial contamination in some operative points of the technology , the detection of contaminants will be attempt from the end-products.

- To apply these adapted and developed methods on an example of a special dietary uses product for celiac patientes.

2. MATERIALS AND METHODS

2.1. Samples

The optimalization was absolved with whole-grain flours getting from different species of wheat, barley, rye, oat, triticale, rice, maize, buckwheat and amaranth which are spreaded in Hungary. The effects of the heat treatment were analysed on bread, pasta and cooked pasta samples.

The adapted and developed methods were testing with further samples. With the helpfull contribution of Dunakenyér Sütőipari és Kereskedelmi Rt. An artificial contamination was performed in a gluten-free bread manufacturing technological line and bread samples were used for further controlling. The raw materials (maize-starch, rice-flour, potato- flakes, egg-powder and yeast) of the technology were also tested.

As a further adaption, the methods were tested on recently developed and introduced yellow-pee noodles made from yellow-pee flour.

2.2. Applied methods

This study is using PCR based technics. Depending on the amount of the samples 2 or 3 paralel izolations were performed with Wizard resin based DNA izolation kit. The characterizatin of DNA solution was performed with spectophotometry and the segmentation of the DNA chain was supervised by agarose gelelectroforesis.

Theadapted primer pairs used for kvalitative polimerase chain reaction were sintetased by right of the sequences characterized in relevant publications. During the primer selection it was an important point of view that the heat treating drastically degradating the DNA chain (under 1000 bp). 4 primer pairs were adapted for this study: A49855 and B49317 chloroplastis DNA primer pair (400-700 bp amplicon size, species-specific) to check the amplicability of the izolated DNA solution, TR01/TR02 wheat-specific primer pair (109 bp amplicon size) for the control of the DNA origin, a glutenin specific microsatellite (P1/P2) primer pair (150 bp amplicon size) for checking the presence of the intron sequence of LMW glutenin gene and finally a wheat, barly, rye specific WBR11/WBR13 primer pair (201 bp amplicon size in case of the wheat and the rye; 196 bp amplicon size in case of the barley). In this last case a PCR-RFLP detection were performed as was characterized in literature. The digestion of the amplicons were performed with restriction endonucleases: *Alu*1 and *Bsm*A1.

During the optimalization the PCR products were visualized and identified on acrilamide gelelectroforesis. For the further application agaroze gelelectroforesis was used as identification method.

3. RESULTS

During the experiment 4 primer pairs were adapted to analyse the different samples: A49855 and B49317 chloroplast DNA primer pair (amplifies 400-700 bp long amplicons, plantspecies-specific) in order to check the amplificability of the isolated DNA; TR01/TR02 wheat-specific primer pair (amplifies 109 bp amplicons) for proving the origin of the DNA; a glutenin-specific microsatellite (P1/P2) primer pair (amplifies 150 bp amplicons) in order to find the specific intron sequence of the LMW glutenin gene; and finally a wheat-, barley- and rye-specific WBR11/WBR13 primer pair (amplifies 201 bp amplicons in case of wheat and rye; amplifies 196 bp amplicons in case of barley) for the selective detection of these three cereals. In case of the last primer pair a PCR-RFLP analysis for PCR-products was adapted regarding the references.

The adapted and developed methods were optimized on different samples like: flours from different varieties of wheat, wheat pastas, boiled pastas, wheat breads, cross-reactive (rye, barley, oat(?), triticale) and not cross-reactive (rice, maize) cereals, pseudocereals (amaranth, buckwheat).

The amplifiability and purity (lack of PCR inhibitors) of the DNA samples was proved by determining the R value and by the amplification with the B49317/A49855 plant-specific primer pair. Considering that this analysis has eventuated species-specific signals, this is a good opportunity for pre-selection of the foodstuffs containing forbidden cereals according to these results.

With the TR01/TR02 primer pair based analysis a conservative wheat DNA sequence was detected in the wheat containing samples. There was no cross-reaction detected with other cereals. Nevertheless, the wheat origin of the DNA samples was also proved in cases of the strongly heat treated samples.

The P1/P2 primer pair was used for identification of the presence of partial sequence from the LMW-glutenin coding gene. It was turned out that this primer pair is applicable to detect both the wheat and the triticale, too. During this analysis the amaranth DNA eventuated a very similar size of amplicon than the wheat and triticale samples. Considering this result the application of amaranth as negative control, may result in a false-positive signal in case of a gelelectroforetogram of inferior quality, therefore its use as a negative control is not recommended.

Finally it was proved also that the adapted wheat-, barley- and rye-specific WBR11/WBR13 primer pair is appropriate for detecting triticale, too. The DNA fragments amplified by this primer pair, according to the references, were digested with Alu1 (effected 68 bp and 133 bp long DNA-

fragments) and BsmA1 (effected 86 bp és 115 bp long DNA-fragments) restriction endonucleases in order to identify and refine the results with PCR-RFLP method.

After an adequate optimization, each methods mentioned above proved appropriate one by one, or more effectively in sequence for detection of gluten contamination. After optimization, the adapted and developed methods were tested on further samples: samples from a factory making gluten-free bread and samples from other gluten-free products (yellow-pea noodles).

With kind contribution of „Dunakenyér Sütőipari és Kereskedelmi" LTD. the analysis of a gluten-free bread manufacturing technology was performed in order to detect the critical contamination points. The raw materials (maize-starch, rice-flour, potato- flakes, egg-powder and yeast) were analysed to exclude the incidental storage failures. An artificial contamination was also performed in 3 operative points of the technology (in the kneader-engine, in the pastry cutters and during desiccation of the gluten-free bread). In two series of production wheat and rye flours were used as contaminants, and then their detection was attempted from the end-products. Through the intervention of exact definition of the critical points, the contamination risk could be easily determined. As a summary it can be concluded that wheat specific PCR method detects even traces of wheat residues, due to the high sensibility of this primer pair. This contamination was detectable also in the end-products. Regarding the other methods like wheat-, barley- and rye-specific PCR it was found that the most critical risk points of the technology were the clearness of the kneader-engine and the place of 24 hour long drying of the gluten-free bread products.

As a further adaption, the PCR methods were tested on recently developed and introduced yellow-pee noodles. Concerning the results it can be pointed out that although the yellow-pea gives no crossreaction with the specific primers of the prohibited cereals, the yellow-pea flour and the yellow-pea noodle samples originating from the production, gave positive contamination signals. It could be probably due to the inadequate milling circumstances or due to the storage failures of the flour on the commercial circulation.

3.1. New scientific results

1. In order to develop method for the controlling of gluten contamination I was adapted B49317/A49855 plant-specific primer pair and TR01/TR02 wheat-specific primer pair as control method. I was appointed that the amplification with plant-specific primer pair is feasible for the selective detection of the prohibited cereals in presence of the other non-toxic cereals and pseudo-cereals, because their amplicon size are considerably similar. At the present prohibited, but not unequivocally toxic oat can be separated with these methods, because of the different amplicon size. The two methods were also applicable on strongly heat-treated samples.

2. With the application of P1/P2 mikrosatellite (SSR) primer pair, I developed a controlling method for detecting gluten contamination, which detects a repetable, non-coding sequence of the LMW-glutenin gene from wheat and triticale.

3. I was specified 7 species of cereals and 2 species of pseudo-cereals with the controlling method based on wheat, rye and barley specific WBR11/WBR13 primer pair and a PCR-RFLP analysis. I was the first, who demonstrate, that except wheat, rye and barley, the technic is applicable for triticale detection. This method was also applicable on strongly heat-treated samples.

4. I was the first, who was screening a gluten free bread manufacturing technology from raw materials to end-products in order to detect the critical contamination points. The samples from the different technological steps were tested with plant-specific, wheat-specific, LMW-glutenin-specific and wheat, rye, barley specific PCR methods. These methods were also tested on recently developed and introduced for special dietary uses yellow-pee noodles. The conclusion was that the contamination of the raw material is influencing the end-product quality, because the contamination is detectable even after the heat treatement.

5. Based on the scientific literature, I was the first, who apply artificial contamination on a manufacturing technology in order to detect the critical contamination points. In two series of production wheat and rye flours were used as contaminants, and then their detection was attempted from the end-products. As a summary it can be concluded that wheat specific PCR method detects even traces of wheat residues. This contamination was detectable also in the end-products. It was found that the most critical risk points of the technology were the clearness of the kneader-engine and the place of 24 hour long drying of the gluten-free bread products. The upper layer contamination of the pastry cutters was not detectable.

4. CONCLUSIONS AND SUGGESTIONS

In the future is referenced these methods to be tested on further commercially available food samples. It would be essencial to prove the results also with ELISA method or other immunoanalitical method in order to find out the corrrelation between DNA and protein, and to characterize the extractability of these biomolecules.

It would be good to design new primers on different sequences from databanks and to check them on different food samples. For most effective methodologies it is required further DNA extraction method developement in order to extract DNA also from the complex food-matrixes.

It would be a new way if a restriction endonuclease digestion (AluI and BsmAI) would be performed with the amplicons of B49317/A49855 primer pair. It would be possible to separate the different species this way.

It would be an important goal to develope or adapt a real-time PCR method in order to be able to find out the kvantitative correlation between DNA and proteins in different samples.
Finally it would be necessary to monitor more food technology to allocate for GMP the critical contamination points.

PUBLICATIONS CONNECTED WITH THE SUBJECT OF THE DISSERTATION

Publication in review

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NÉMEDI E., UJHELYI G., GELENCSÉR É.(2007): Detection of gluten contamination with PCR method. *Acta Alimentaria* Vol. 36. (2), pp. 241-248.

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