



**INVESTIGATION OF COMMON BEAN DERIVED BIOACTIVE
ALPHA-AMYLASE INHIBITORS**

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1. INTRODUCTION

Food legumes are valuable sources of dietary proteins, carbohydrates, fibres, minerals, vitamins and play important, diverse role in the human nutrition and animal feeding. They contain a number of bioactive compounds that are not nutrients, but exert metabolic effects on human and animal health. These compounds have long been classified as antinutritional factors, but recent studies have reconsidered their impact on health and demonstrated that these antinutritional compounds, like lectins and protease inhibitors may have beneficial properties also. These aspects have received increased attention in the areas of food science and nutrition and have been associated with beneficial health-promoting properties, like managing high cholesterol and type-2 diabetes and in the prevention of cancer. The modern biotechnology also exploits these compounds in the plant defence strategies.

Common beans (*Phaseolus vulgaris*) contain bioactive alpha-amylase inhibitors (α AI), which reduce amylase activity and starch digestion. The group α AI-1 has been shown to inhibit human and insect derived pancreatic and salivary alpha-amylases. The α AI-1 is derived from a pre-protein, of which the native or unprocessed molecular weight has been estimated to be around 28 kDa. It is synthesized on the endo-plasmic reticulum and after the cleavage of the signal peptide, glycans are added in the Golgi apparatus which leads to an increase in the molecular weight of the pre-protein to 32-36 kDa. Finally the pre-protein is transported to protein storage vacuoles, where proteolytic cleavage results into two subunits (α -and β -chains) of the inhibitor.

More recently, common bean derived alpha-amylase inhibitor-1 (α AI-1) has been tested in clinical studies for its application in weight loss and glycemic control as they delay carbohydrate absorption. However, clinical studies have shown that crude preparation of bean α AI-1 had no inhibitor-activity effect, while a partially purified bean product was found to indicate body weight loss and reduce spikes in blood sugar due to its α AI-1 activity. Up to now there is not enough experimental data to understand why a less purified preparation of α AI-1 can be more effective physiologically. To understand the mechanism of this action requires further researches.

As the α AI-1s play an important role in the starch digestion of animals, plants and microorganisms, the modern biotechnology has utilized this property in the plant protection against insects. The introduction of the bean (*Phaseolus vulgaris*) α AI-1 gene in pea (*Pisum*

sativum) leads to expression of seed-specific α AI-1 protein accumulated at high level and undergoes post-translational modification. This proved to be a good strategy for protection of the plant from damage by pea weevil (*Bruchus pisorum*) under greenhouse and field growth conditions as well. However, a study demonstrated that the α AI-1-expressing pea led to the synthesis of a modified form of the protein with altered immunogenicity. This modification was thought to be responsible for the reported allergenicity in mice of the transgenic pea but not the bean. The authors presumed that the differences are due to post-translational modifications. Further investigations revealed some heterogeneous structural variations in genetically modified pea and bean due to differences in glycans, but no evidence was found for the increased immunogenicity of the α AI-1 in pea. The α AI-1s from the two sources can induce similar immune responses, both α AI-1 forms elicited Th1 and Th2 antibody responses, suggesting that both forms can be immunogenic. Therefore the allergenic effect of the α AI-1 is still controversial.

Most of the proteins are hydrolyzed in the gastrointestinal tract, but some bioactive proteins can show resistance against the digestive enzymes. There are informations about the effects of bean α AI-1 on the digestion, but experimental data are missing so far on the digestive fate of bean α AI-1. Many studies oriented to the assessing of structure-function relationship of the α AI-1, but further accumulation of the data is still required for the better understanding, especially the variability assessment of the bean α AIs and its transgenic expression. The proteomics investigation of the protein is reasonable for nutrition and food safety aspects.

2. AIMS

- Characterize α AI-1s obtained from Hungarian *Phaseolus vulgaris* bean varieties in comparison with α AI-1 obtained from *Phaseolus vulgaris* L. cv. Tendergreen and a genetically modified pea (*Pisum sativum* L.) expressing α AI-1 regarding their structures and functions.
- Investigate the IgE-reactivity of the Tendergreen bean and the GM pea α AI-1s using sera obtained from severe combined immunodeficient mouse model in comparison with clinically proved legume allergic human sera.
- Describe the difference in the digestive fate of common bean α AI-1 released from the purified protein and the crude protein extracts using simulated gastric fluid and acute rat digestion model.

3. MATERIALS AND METHODS

3.1. Common beans and isolated proteins

Reference materials (Bean and pea meals and isolates provided by the Australian CSIRO (Higgins T.J.W.) institute in the framework of *EU FP7 GMSAFOOD* project):

- Donor Tendergreen bean meal (*Phaseolus vulgaris* L. vs Tendergreen, TG) and purified α AI-1 (MARSHALL and LAUDA, 1975) in lyophilised form
- Genetically modified (GM) pea meal (*Pisum sativum* L.), which is a product of an *Agrobacterium*-mediated transformation (SCHROEDER et al.,1995) containing a chimera α AI-1 gene (SHADE et al. (1994) and purified α AI-1 (MARSHALL and LAUDA, 1975) in lyophilised form
- Non GM (nGM) pea meal (*Pisum sativum* L.)
- Commercial pea meal (*Pisum sativum* L.)

Common beans obtained from Hungarian trade:

- Huanita- Dry bean (*Phaseolus vulgaris*)
- Bushbean (*Phaseolus vulgaris*)
- Red-Kidney bean (*Phaseolus vulgaris*)
- Pinto bean (*Phaseolus vulgaris*)
- Purified α AI-1s from the Hungarian beans and the GM pea

As my thesis is extended to the α AI-1, hereafter I use the α AI notation.

3.2. Sera

- Clinically proved legume allergic anonym human sera (Allergo-Derm Bakos Kft. Validation Number of Ethical Committee: GH277-1/2011). Patients with chronic urticaria or unrecognized urticaria symptoms and in some cases respiratory symptoms in response to legumes were compared with healthy controls without allergy. Patients included in the study had clinical symptoms after legume exposure and positive allergen specific *Prick in Prick* test to native legumes. The score values were related to the severity of the clinical

symptoms (1:+, 2: ++, 3: +++). After 4 weeks on elimination diet and repeated legume food challenges severe clinical symptoms have been observed.

- Sera obtained from HuSCID mouse model (Provided by the Medical University of Vienna Austria (MUW), in the framework of the *EU FP7 GMSAFOOD* project, (LEE et al., 2013). The HuSCID mice model was developed by Medical University of Vienna, Severe combined immunodeficiency (SCID) mice lacking functional T- and B-cells were reconstituted with human peripheral blood mononuclear cells (PMNCs). PMNCs were isolated and transferred from healthy individuals and selected legume allergic patients. The Hu-SCID mice developed allergic asthma upon feeding with GM peas and Tendergreen beans and challenge with pure α AI.
- Polyclonal antibody against Tendergreen bean α AI produced in rabbit (anti- α AI rabbit IgG). Developed at the Unit of Biology in the NAIK-ÉKI (Dr. Nagy András, Dr. Takács Krisztina) in the framework of the *EU FP7 GMSAFOOD* project (HARBOE and INGILD, 1973)

3.3. Methods

- Meal of the Hungarian beans and GM pea were ground to a fine powder, stirred with distilled water in 1:10 ratio (w/v) for 1 h at room temperature and centrifuged at 10000 g for 10 minutes.
- The electrophoresis separations in denaturation conditions (SDS-PAGE) were carried out by the method of LAEMMLI (1970) using 15% separation gel and 6% stacking gel at 200 V, 400 mA in MINI-PROTEAN 3-CELL (Bio-Rad) equipment. The gels were fixed in 20% TCA (trichloroacetic acid) and stained with Commassie Brilliant Blue R-250.
- For native-PAGE 10 % gel was used at 200 V, 47 mA. After separation the gels were incubated in alpha-amylase enzyme and starch containing buffers in room temperature. The active α AI bands were visualized by potassium iodide solution.
- The first dimension of the two-dimensional electrophoresis (2-DE) was the isoelectric focusing, which was carried out by linearly increasing voltage from 250V to 24.000 Vh using a commercial IPG strip, 7 cm long and with a pH range of 3-10. The strips were incubated in DTT and iodoacetamide containing equilibrating solution for 15 min at room temperature with gentle

shaking. The second dimension was carried out on 15% SDS-PAGE with 1 h run at 200 V in a Bio-Rad Protean appliance.

- The lab-on-a-chip (LOC) electrophoresis was carried out by the instructions of the manufacturer.
- The separated α AI proteins were transferred onto a 0.45 μ m PVDF membrane for 1.5 hour at 0.25 V, 0.08 mA/ cm² (Bio-Rad Trans Blott CD Semi-Dry Transfer Cell). The membrane was blocked with 1 % BSA for 40 min and incubated overnight with α AI-rabbit IgG antibody. Peroxidase conjugated anti-rabbit IgG and anti-human IgE were used as secondary antibodies. The blots were developed with 4-chloro-1-naphthol solution containing 0.48 mM 4-chloro-1-naphthol, 50 mM Tris-HCl, 0.2 M NaCl and 17% methanol.
- The deglycosylation of proteins were carried out by the method of WOODWARD (1984), where the carbohydrates were eliminate from the protein chains by sodium-metaperiodate in acidic conditions.
- The Schiff-staining was carried out by the method of SACCHARIUS (1969). The proteins were incubated in periodic acid, Schiff-reagent and metabisulfite.
- For protein purification DEAE-anion-exchange and Superose 12 gel-filtration columns were used.
- The α AI activity was determined by the method of MURAO (1981), where the protein solution was incubated with porcine pancreatic alpha-amylase enzyme and starch. The reaction was stopped by acidic solution and incubated in iodide solution. The absorbance was measured at 660 nm, from which the activity was determined by the following:
Inhibitory activity (%) = $\frac{OD_{\text{sample}} - OD_{\text{control}}}{OD_{\text{blind}} - OD_{\text{control}}} \times 100$
- The haemagglutination activity was determined by the modified method of CUADRADO (1996). Protein extracts were prepared from the bean and pea meals in 0,9% NaCl solution. From the extracts 100 μ l was pipetted onto the microtiter plate and 100 μ l of rat blood containing EDTA were added to the samples. After incubation at room temperature the samples were correlated to control (bean PHA-L) visually. The Tendergreen bean and GM pea derived α AI were used in the same way in 1 mg/ml concentration.
- For mass-spectrometry, the 2-DE separated α AI spots were excise from the gels and digested by trypsin. The digested samples were injected (2 μ L) on a Ultimate 3000 RSLC (Dionex, Germany) equipped with a Zorbax 300 SB-C8 column (3.5 micron, 2.1x150mm, Agilent Technologies) applying a flow rate of 0.2 mL/min. The column was operated at a constant temperature of 40°C. A gradient between 0.1% acetic acid in 90% water/10%

acetonitrile (solvent A) and 0.1% acetic acid in 10% water/90% acetonitrile (solvent B) was applied at the following gradient: 10 % B; 1-11 min: 10-100 % B; 11-16 min: 100 % B; 16-16.5 min: 100-10 % B and from 16.5-21 min: 10 % B. The eluted peptides were analysed by a UV detector (Dionex, Germany) at 214, 280 nm and by the micro-TOF II time-of-flight mass spectrometer (Bruker Daltonics, Germany). The electrospray ionization source was operated in positive mode; the nebulizer (N₂) pressure was set at 2 bars, the nebulizer (N₂) gas flow at 4 L/min. and the dry temperature at 200°C. The capillary voltage was maintained at 5800 V, the capillary exit voltage at 150 V, the skimmer potential was 50 V and the hexapole RF was set at 150 V. Conventional ESI-MS data were recorded using a scan range of m/z 100 to 3000 and screened for the masses of the α AI peptides which were obtained after an *in silico* tryptic digestion of α AI from *Phaseolus vulgaris* (P02873, UNIPROT). Since it is known that electrospray ionization of tryptic peptides results in predominantly doubly charged ions, $[M+2H]^{2+}$, a screening for respective masses was performed.

- *In vitro* simulating gastric fluid (SGF) assay was performed by the protocol of THOMAS et al. (2004) and KUMAR et al. (2011) with some modifications. Hence, 90 μ L of pure α AI (5 mg/ml) or 75 mg bean meal was dissolved in 1,5 ml SGF. The samples were added to the SGF (3.2 mg porcine pepsin, 30 mM NaCl, 2 μ L 4M CaCl₂) and incubated in water bath at 37 °C. After the digestion process the immunoreactivity was investigated on immunblot and the inhibitory activity on native-PAGE.
- To monitor the α AI during the gastrointestinal digestion *in vivo* acute rat model was used. Followed 60 min digestion the stomach and small intestinal contents were removed. Proteins were extracted from the lyophilised stomach and small intestinal content and immunoreactivity against Tendergreen bean α AI was determined by immunblot and inhibitory activity was determined by native-PAGE. The proteins extracted from the stomach samples were analysed by LOC separation as well.

4. RESULTS

- According to the first objective, my results obtained by proteomics contributed to the understanding of biochemical properties of α AIs found in Hungarian common bean varieties. Differences were observed in the presence of α - and β -chain peptides in the different α AIs using LC-TOF-MS method. As the detection based on the tryptic digested peptide markers of the Tendergreen bean α AI, therefore carrying-on the work it is need to determine the amino acid sequences of the Hungarian bean derived α AIs as well.
- In the SDS-PAGE separated Hungarian common bean proteins and the GM pea proteins α AI bands were identified by anti α AI polyclonal antibody produced in rabbit. The separated bean and pea proteins showed inhibitory activity as well on native -PAGE, which was proved also by spectrophotometric method.
- While on 2-DE protein map the nGM pea meal contained 54 protein spots, the GM pea meal contained 79 protein spots.
- The bean and pea protein extracts lost the majority of their inhibitory activity after 10 min heat-treatment on 80 °C and after 10 min on 100°C this activity was totally lost.
- The purified α AIs did not showed hemagglutination activity, contrary to the bean and pea protein extracts.
- Cinc-hydroxide adsorbent based new chromatographic method was developed for α AI purification, by which α AIs were isolated from the Hungarian bean varieties and the GM pea.
- I confirmed that the common bean derived α AIs contain the pre-protein of the α AI contrary to the GM pea derived isolates by electrophoretic (1-DE, 2-DE, LOC) and mass-spectrometric (LC-ESI-TOF-MS) methods.
- I verified that the α AI-s are glycoproteins by Schiff-staining.
- I detected three protein spots of the α AI's obtained from the *P. vulgaris* varieties and two spots of the GM pea α AI by 2-DE.
- According to the second objective, I carried out immunblot methods for the assessment of the potential allergency of the α AI.
- On the basis of the amino acid sequence of the common bean α AI using FAO/WHO criteria containing database (SDAP, ADSF, ProAP) I screened known allergens showing sequence homology to the α AI.

- Using epitope prediction software (IEDB) I mapped potential linear B-cell epitopes on the amino acid sequence of the α AI.
- Using clinically proved legume allergic anonym human sera I selected α AI specific IgE reactive sera by immunoblot, which confirmed the potential allergenic property of the bean derived α AI. By deglycosylation of the protein I excluded the carbohydrate-specific IgE binding.
- I found similar IgE recognition patterns of the sera obtained from human patients and sera obtained from HuSCID (humanised severe combined immunodeficient mice) model proved by the GMSAFOOD partners.
- According to the third objective, using simulated gastric fluid, I showed immunoreactive α AI bands after 60 min *in vitro* pepsin digestion, but the inhibitory activity was not detectable in contrast to the raw bean protein extract, which was still active after the digestion process.
- Using acute rat digestion model I had similar observations. The protein survived the passage through the stomach in inhibitory active form and it was able to form an enzyme-inhibitor complex in the small intestine since its activity was not detectable anymore. Even a heat-treated Pinto bean extract proved to be active against the amylase-enzyme in the gastrointestinal tract.

5. NOVEL SCIENTIFIC RESULTS

1. Using zinc-hydroxide inorganic adsorbent I developed a new, shortened chromatographic method for α AI purification. By this method I isolated α AI from four Hungarian common bean varieties (Huanita Dry bean, Bush bean, Red Kidney bean, Pinto bean) and I detected their α AI activities.
2. In common bean and GM pea derived α AIs I showed unprocessed, Schiff-stain negative pre- α AIs showing cross-reactivity with antibody produced against Tendergreen bean α AI. I verified the variability of the unprocessed alpha-and beta-chains of the α AI.
3. In common bean and GM pea derived α AIs I identified processed, glycosylated iso-proteins at the 13-18 kDa molecular weight region, at 4.7-5.1 and 5.7-6.0 isoelectric point region. I detected the variability of the alpha-and beta chains in the processed forms as well.
4. I proved the α AI specificity in clinically proved legume allergic anonym human sera by HuSCID mouse model, which is a novelty in the field of allergy research.
5. I showed immunoreactivity and partial inhibitory-activity of common bean and GM pea derived α AIs in simulated gastric fluid followed by *in vitro* pepsin digestion, which was presumably caused by the activation of the pre- α AI during the digestion process.
6. I proved that after *in vivo* digestion of a 100 °C heat-treated Pinto bean protein extract the α AI is detectable in immunogen and inhibitory- active form in the stomach, while the activity was lost in the small intestine.

PUBLICATIONS RELATED TO THE SUBJECT OF THE PhD DISSERTATION

Articles in journals with impact factor

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R. Lee, D. Reiner, **A. Maczó**, N. Bakos, É. Gelencsér, T. J. V. Higgins, M. M. Epstein (2013): Using human severe-combined immunodeficiency (Hu-SCID) mice as a model for testing allergenicity of genetically modified organisms (GMOs). Food Allergy and Anaphylaxis Meeting, 7 – 9 February 2013., Nice, France. *Clinical and Translational Allergy* 2013, 3 P80 DOI:10.1186/2045-7022-3-S3-P80

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