Food and Chemical Toxicology 76 (2015) 54–60

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Enzymatic hydrolysis: A method in alleviating legume allergenicity

Ramkrashan Kasera [a], A.B. Singh [a], S. Lavasa [b], Komarla Nagendra Prasad [c], Naveen Arora [a,*]

[a] Allergy and Immunology Section, CSIR-Institute of Genomics and Integrative Biology, Delhi 110007, India
[b] USLavasa Medical and Research Center, Chandigarh 160015, India
[c] Bengaluru Allergy Centre, Bangalore 560027, India

ARTICLE INFO

Article history:
Received 8 June 2014
Accepted 26 November 2014
Available online 3 December 2014

Keywords:
Enzymatic hydrolysis
Food allergy
Histamine
Legumes
Skin prick test

ABSTRACT

Legumes are involved in IgE mediated food allergy in many countries. Avoidance of allergenic food is the only way to avoid symptomatic reaction. The present study investigated the effect of enzymatic hydrolysis on the allergenicity of three legumes – kidney bean (*Phaseolus vulgaris*), black gram (*Vigna mungo*) and peanut (*Arachis hypogaea*). Soluble protein extracts of the study legumes were sequentially treated by Alcalase® and Flavourzyme®. Allergenicity of hydrolysates was then determined by ELISA, immunoblot, stripped basophil histamine release and skin prick test (SPT). Hydrolysis resulted in the loss of all IgE binding fractions determined by immunoblot in the three legumes. Specific IgE binding in ELISA was reduced by 62.2 ± 7.7%, 87.1 ± 9.6% and 91.8 ± 7.2% in the hydrolysates of kidney bean, black gram and peanut, respectively ($p < 0.01$). The release of histamine was decreased significantly when sensitized basophils were challenged with hydrolysates as compared to raw extracts. Significant reduction in the biopotency of hydrolysates was also observed in SPT where only 1/10 kidney bean-sensitive individuals, 2/6 black gram-sensitive individuals and 1/7 peanut-sensitive individuals were found positive to their respective hydrolysates. In conclusion, enzymatic hydrolysis is effective in attenuating allergenicity of legume proteins and may be employed for preparing hypoallergenic food extracts.

1. Introduction

Legumes are a rich source of proteins and have significant nutritional value. In combination with cereals, legumes also provide necessary amino acids (Bucci and Unlu, 2000). However, many legumes can cause permanent or fatal food allergies in sensitive individuals (Bock et al., 2001; Rougé et al., 2011; Sicherer and Sampson, 2009; Zacharisen and Kurup, 1998), are an important risk factor for asthma in children (Roberts et al., 2003; Wang et al., 2005), and are among the most common foods causing allergic reactions among children in the Mediterranean region (Martínez San Ireneo et al., 2000; Pascual et al., 1999; Patil et al., 2001). The legumes most commonly implicated in allergic reactions are peanut (*Arachis hypogaea* L.), soybean (*Glycine max* (L.) Merr.), lentil (*Lens culinaris* Medikus), chick pea (*Cicer arietinum* L.), black gram (*Vigna mungo* (L.) Hepper) and pigeon pea (*Cajanus cajan* (L.) Millsp.) (Cordle, 2004; Ibáñez et al., 1999; Kumari et al., 2006; Patil et al., 2001).

Many studies have evaluated the effects of different processing methods on food proteins which could decrease or eliminate their allergenicity (Merritt et al., 1990; Terracciano et al., 2002). Several approaches have been used to reduce the allergenicity of food proteins e.g. heat treatment, γ-radiation, enzymatic hydrolysis, gene manipulation, RNA interference, etc. Efforts are being made to process the foods in such a way which may enable hypersensitive individuals to have nutrition from such foods without having any adverse or hypersensitive effect (Riascos et al., 2010; Sicherer and Sampson, 2006; Zhenxing et al., 2007). Among these processing methods, heat treatment has been the preferred method of choice for altering the allergenicity of food proteins. Heat changes the solubility, stability, size, and compactness of protein, which attributes to the structural folds of the protein and hence to its allergenicity (Aalberse, 2000). It is also known that the conformational epitopes show lesser affinity to immunoglobulin E (IgE) epitopes of food allergens after cooking or partial hydrolysis of food (Sampson, 2004). This is because the tertiary structure of the protein is altered leading to the destruction of conformational epitopes. On the other hand, IgE binding to linear epitopes remains unchanged as the linear sequence of the amino acids is generally unaltered by denaturation (Lee et al., 2007). Since the number of allergic incidences due to legumes is increasing (Kasera et al., 2011), the production of safe and effective hypoallergenic diets on a commercial scale has become a major interest. Although elemental diets with free amino acids are allergen-free and can be used with success in therapy, their long term use may cause osmotic problems. Thus, protein hydrolysates that show reduced antigenic activity are provided in hypoallergenic formulas for susceptible infants (Alexander and Cabana, 2010; Knipping

* Corresponding author. Allergy and Immunology Section, CSIR-Institute of Genomics and Integrative Biology, Mall Road, Delhi University Campus, Room 509, Delhi 110007, India. Tel.: +91-11-27666156 (ext 150); fax: +91-11-27667471.
E-mail address: naveen@igib.res.in (N. Arora).

http://dx.doi.org/10.1016/j.fct.2014.11.023
0278-6915/

et al., 2012). Several investigators have evaluated the change in immunogenicity or allergenicity of enzymatically-hydrolyzed proteins for potential use in formulas for feeding food-allergic individuals (Cordle, 1994). Protein hydrolysates containing di- and tripeptides are absorbed more rapidly than free amino acids (Di Pasquale, 1997). Protein hydrolysates are produced primarily from purified protein sources by heating with acid or addition of proteolytic enzymes followed by purification procedures (Bucci and Unlu, 2000). Enzymatic hydrolysis has been preferred over acid hydrolysis as acid treatment lowers protein quality and its biological value. Acid hydrolysis leads to oxidization of cysteine/methionine, destruction of serine/threonine and conversion of glutamine and asparagine to glutamate and aspartate, respectively. Enzymatic protein hydrolysates constitute an alternative to intact proteins. Special formulations can thus be designed and developed to provide nutritional support to specific population groups such as infants, the elderly and food-allergic patients (Burks et al., 1992; Cabanillas et al., 2012; Clemente et al., 1999; Lee et al., 2007; Merritt et al., 1990; Terracciano et al., 2002). The combination of enzymatic treatment coupled with post-hydrolysis food processing, such as heat treatment and ultrafiltration, are considered to be effective in obtaining protein products for human nutrition and reduced risk of allergenicity (Cabanillas et al., 2012; Clemente et al., 1999).

The present study was therefore aimed to investigate the effect of sequential action of Alcalase® (endoprotease) and Flavourzyme® (exoprotease) on the allergenicity of legumes namely – kidney bean, black gram and peanut using *in vitro* and *in vivo* approaches. The legumes selected for the study are common dietary ingredients and are therefore, potential source of protein hydrolysates for hypoallergenic food extracts.

2. Materials and methods

2.1. Study subjects

Allergic rhinitis and asthma patients with confirmed history of allergy to legumes were included in the study from the two centres; Bangalore Allergy Centre, Bangalore and USLavasa Medical and Research Center, Chandigarh, India. The diagnosis of asthma and rhinitis was ascertained as described in previous studies (Kasera et al., 2011, 2012). Patients showing skin prick test (SPT) positivity to kidney bean, black gram and peanut with symptoms such as anaphylaxis, redness of mouth, urticaria, nausea, vomiting, diarrhoea, abdominal cramps, runny nose or breathlessness after ingestion of legumes were recruited for the study. Blood was collected from patients who showed the reversal of their respective symptoms after elimination of suspected study legumes from their diet (n = 30). Blood was also collected from healthy non-allergic individuals (controls, n = 5). The study protocol was approved by Human Ethics Committee of Institute of Genomics and Integrative Biology, Delhi. Informed written consents were obtained from all the patients and controls participating in the study.

2.2. Preparation of extracts

The extraction of study legume proteins was carried out following the protocol described earlier (Kasera et al., 2013). Briefly, kidney beans were crushed and defatted in diethyl ether at 4 °C. The antigens were extracted in 1:20 (w/v) ammonium bicarbonate buffer (50 mM, pH 8.0) with 5 mM EDTA and 1 mM phenyl methyl sulphonyl fluoride by continuous stirring for 8 h at 4 °C. The supernatant was dialyzed using 0.05 M PBS pH 8, passed through 0.22 μm nitrocellulose membrane filters and lyophilized. The extracts for peanut and black gram were prepared similarly. Protein was estimated in the extract using modified Lowry's method (Singh et al., 1992).

2.3. Enzymatic treatment (hydrolysis)

The protein extracts of study legumes were hydrolyzed according to the method of Clemente et al. (1999) with small modifications. The enzymes used for producing hydrolysates were Alcalase® and Flavourzyme® (Sigma Aldrich Co., USA). The raw extracts of the study legumes were hydrolyzed with Alcalase® and Flavourzyme® by sequential treatment. Initial hydrolysis for 180 min was performed using Alcalase® followed by Flavourzyme® for another 300 min (180 to 480 min). For hydrolysis using Alcalase®, 400 mg of legume protein with a protein concentration of 20 mg/mL was mixed with 33.6 μg of Alcalase® enzyme at a temperature of 50 °C and stirred continuously for 3 h with constantly maintained pH of 8.0 for the reaction mixture. Samples (1 mL) were withdrawn at 0, 15, 30, 60, 90, 120, 150 and 180 min.

After taking 8 aliquots at 8 different time intervals after initial digestion with Alcalase® 12 mL of Protein (240 mg) was left to which 12 μg of the Flavourzyme® was added for sequential digestion. Continuous stirring for 5 h was done with constantly maintained pH (8.0) and temperature (50 °C). Samples (1 mL) were withdrawn at 210, 240, 270, 300, 330, 360, 390, 420, 450 and 480 min. Proteases in the aliquots were inactivated by heating at 80 °C for 20 min.

The degree of hydrolysis (DH), defined as the percentage of peptide bonds cleaved, was measured by determination of free amino groups by reaction with tri-nitrobenzenesulphonic acid (Sigma Chemicals, USA) with leucine as positive control, according to the method of Adler-Nissen (1979). The total number of amino groups (total hydrolysis) was determined in the extract after acid hydrolysis using 6N HCl at 120 °C for 24 h. This gives the O.D. of total amino groups present after total hydrolysis. Similarly, the value (O.D.) of free amino groups in raw extracts and hydrolysates (taken at different time intervals) were determined and degree of hydrolysis was calculated using the following reaction.

$$\text{Degree of hydrolysis} = \frac{\text{Total hydrolysis (acid)} - \text{Enzymatic hydrolysis}}{\text{Total hydrolysis (acid)}} \times 100$$

2.4. Skin prick tests

SPT were performed with raw legume seed extracts (1:10 w/v) of kidney bean, black gram and peanut reconstituted in 50% glycerinated phosphate buffered saline (PBS). Histamine diphosphate (5 mg/mL) and PBS in 50% glycerine were used as positive and negative controls, respectively. The SPT reactions were observed after 20 minutes and positivity was graded from 1 + to 3 + based on wheal diameters of tests and controls (Kumari et al., 2012). Some of the SPT-positive patients were also skin-tested later with the legume hydrolysates for comparing the *in vivo* effect of enzymatic hydrolysis.

2.5. SDS-PAGE and immunoblotting

Extracts of legumes were loaded on 12% SDS-PAGE along with their hydrolysates obtained after enzymatic treatment for different time intervals as described previously (Kasera et al., 2013). The resolved gels were stained and visualized with Coomassie brilliant blue.

The resolved protein were transferred onto nitrocellulose membrane for immunoblotting as described by Kasera et al. (2013) and pooled patients' sera were used for the identification of IgE binding proteins in different legume extracts and respective hydrolysates.

2.6. Estimation of specific IgE

Serum-specific IgE with legume extracts and the hydrolysates (8 h) was determined by ELISA using individual patient's sera. Briefly, hydrolyzed (8 h) and raw extracts of legumes were coated on microtitre plates. After blocking with 1% BSA, wells are incubated with the respective serum samples. The plate was washed and incubated with antihuman IgE–horse radish peroxidase (1:1000 v/v; Southern Biotech, USA). Colour was developed with orthophenylene diamine. The reaction was stopped by adding 2.5 M H_2SO_4 and the absorbance was read at 492 nm (Kasera et al., 2012).

To determine time-dependent change in IgE binding of different hydrolysates, specific IgE was also estimated using hydrolysates obtained at 0, 15, 30, 150, 180, 210, 390 and 480 min using pooled patients' sera.

2.7. Stripped basophil histamine release assay

Histamine release assay was performed with 10 patients sensitive to kidney bean, black gram and peanut each. Patients' sera were used in the protocol described earlier (Kasera et al., 2013). Briefly, peripheral blood was drawn from non-allergic subjects and basophils were separated using centrifugation. The basophils in suspension were stripped off bound IgE by incubating the cells for 3.5 min with lactic acid buffer (13.4 mM lactic acid, 140 mM NaCl, 5 mM KCl, pH 3.9). After stripping basophils were resensitized with serum IgE of individual patients and controls. The optimal histamine release was determined for each study legume by testing different concentration of protein for challenge (data not shown). The protein concentration inducing optimal histamine release (10 ng for kidney bean, 35 ng for black gram and 10 ng for peanut protein) was selected for the assay. After passive sensitization, cells were stimulated with respective raw legume antigens and hydrolysates for 1 h. The histamine content was determined as described earlier (Kasera et al., 2013).

2.8. IgE inhibition assay

The allergenic potency of crude legume extracts along with respective hydrolysates (3 and 8 h) were determined by ELISA inhibition (competitive ELISA) using hypersensitive pooled patients' sera as described previously (Kasera et al., 2011). Briefly, kidney bean extract (1 μg/100 μL/well) was coated in carbonate buffer overnight at 4 °C on a microtitre plate. The kidney bean patients' pooled serum