

FULL ARTICLE

Anti-allergic activity of mung bean (*Vigna radiata* (L.) Wilczek) protein hydrolysates produced by enzymatic hydrolysis using non-gastrointestinal and gastrointestinal enzymes

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Abstract

Mung bean seed is a well-known plant protein consumed in Asian countries but the protein is usually retrieved as a waste product during starch production. This study investigated the anti-allergic property of mung bean protein hydrolysates (MBPH) produced by enzymatic hydrolysis using non-gastrointestinal (non-GI), GI and a combination of non-GI+GI enzymes. The hydrolysates were investigated for any anti-allergic property by detecting the amount of β -hexosaminidase released in RBL-2H3 cells, and complemented with the MTT assay to show cell viability. It was found that MBPH hydrolyzed by a combination of flavourzyme (non-GI enzyme) and pancreatin (GI enzyme) exhibited the highest anti-allergic activity (135.61%), followed by those produced with alcalase, a non-GI enzyme (121.74%) and 80.32% for pancreatin (GI enzyme). Minimal toxicity (<30%) of all hydrolysates on RBL-2H3 cells line was observed. The results suggest that MBPH can potentially serve as a hypoallergenic food ingredient or supplement.

Practical applications

Mung bean (*Vigna radiata* L. (Wilczek)) is also known as “green gram” and it is an excellent source of protein. The major mung bean storage proteins are the globulin, albumin and legumin, which are also referred to as legume allergens. Our study showed that mung bean peptides obtained after enzymatic hydrolysis influenced β -hexosaminidase inhibition without any toxic effect on RBL-2H3 cells. This indicates that mung bean allergenicity can be reduced after enzymatic hydrolysis and the protein hydrolysates could be labelled as a hypoallergenic food, ingredient, supplement and/or protein substitute in the formulation of food products.

KEYWORDS

anti-allergic, gastrointestinal enzymes, mung bean protein hydrolysates, non-gastrointestinal enzymes, peptides sequence, RBL-2H3, β -hexosaminidase

1 | INTRODUCTION

Mung bean is a member of the legume family that is commonly consumed in Asian countries and the seed contains about 20.8–28.5% protein content (Abbas & Shah, 2007; Li, Shu, Yan, & Shen, 2010). The major seed proteins of mung bean are storage globulins of vicillin type (8S; ~90%), legumin type (11S; ~8%) and the basic 7S type (~3%) (Mendoza, Adachi, Bernardo, & Utsumi, 2001). The 8S globulins have molecular weight of about 150 kDa (Misra et al., 2011; Mittag et al., 2005). Four proteins were identified from mung bean seeds as follows: Vig r1 (pathogen related protein PR-10) Vig r2 (8S β isoform, 52 kDa), Vig r3 (8S α , 8S α , 50 kDa) and Vig r4 (seed albumin, 30 kDa). Their protein and amino acid sequences exhibit similarity with known allergens (Codina, Lockey, Fernández-Caldas, & Rama, 1999).

The big 8 categories of food allergy consists of eggs, milk, fish, wheat, shellfish, tree nuts, peanut and soy (Sampson, 2004). Lack (2012) found that the prevalence of allergy varied 1.2–17% in milk, 0.2–7% in egg, 0–10% in shellfish, 0–2% in peanut, and 3–35% for general food allergy. Legume allergens are stable in gastric fluid (Astwood, Leach, & Fuchs, 1996) and the allergic effect is an IgE-mediated immunological reaction. The entry of an allergen into the human body elicits secretion of immunoglobulin E (IgE) antibody by B-cell lymphocytes (Janeway et al., 2001). Basophils and mast cells are two functionally similar but distinct cell types that play pivotal roles in the initiation and development of type I hypersensitivity reactions (Gibbs, Amon, & Pearce, 1997). RBL-2H3 cells, like mast cells and basophils, respond with degranulation, which is a cellular process that involves the following steps. Following the crosslinking of their IgE-bound to the alpha subunit of the human IgE receptor (Fc ϵ R1) by multivalent allergens, they release a range of preformed and newly synthesized mediators such as cytokines, histamine and a protease (β -hexosaminidase) that evoke potent immune-based allergic responses (Knapp et al., 1996; Passante & Frankish, 2009). The β -hexosaminidase released from mast cell degranulation is a marker for determining the degree of allergic reaction.

Several studies have evaluated the effect of different processing methods on food proteins and their ability to reduce or eliminate allergenicity such as heat treatment (Riascos et al., 2016; van Boxtel, Broek, Koppelman, & Gruppen, 2008); high pressure treatment (Penas, Préstamo, Polo, & Gomez, 2006), and enzymatic hydrolysis (Cabanillas et al., 2010). Enzymatic hydrolysis is widely applied to destroy or reduce protein allergen in legume seeds such as chickpea (Clemente, Vioque, Sanchez-Vioque, Pedroche, & Millán, 1999), lentil (Cabanillas et al., 2010), and peanut (Yu, Ahmedna, Goktepe, Cheng, & Maleki, 2011). Several proteases have been utilized in the reduction of allergens found in legumes. For instance, chickpea 11S globulin was sequentially hydrolyzed with alcalase and flavourzyme, and the hydrolysates had 90% reduction in allergenicity (Clemente et al., 1999). Also, kidney bean, black gram and peanut protein hydrolysates obtained by sequential hydrolysis with alcalase and flavourzyme resulted in the loss of all IgE binding fractions (Kasera, Singh, Lavasa, Prasad, & Arora, 2015). Furthermore, lentil allergenicity decreased substantially after sequential hydrolysis with alcalase and flavourzyme (Cabanillas

et al., 2010). In previous reports, pepsin and chymotrypsin were used to hydrolyze soybean 2S and 11S protein storage protein (Lee et al., 2007; Sung, Ahn, Lim, & Oh, 2014), while pepsin and porcine gastric fluid were used for peanut (Kopper et al., 2004).

In addition, individual proteases have been used singly to reduce allergenicity such as hydrolysis of glycinin and β -conglycinin with pepsin (Zhao, Qin, Sun, Zhang, & Wang, 2010), peanut with trypsin (Yu et al., 2011), peanut with chymotrypsin (Yu et al., 2011), as well as soy-meal and soybean with alcalase (Tsumura, Kugimiya, Bando, Hiemori, & Ogawa, 1999; Wang et al., 2014). Therefore, we hypothesized that the use of enzyme combinations will be more effective than single enzymes in producing hypoallergenic protein hydrolysates. The aim of this work was to investigate the effect of non-gastrointestinal enzymes (non-GI) such as alcalase and flavourzyme, gastrointestinal enzymes (GI; pancreatin, pepsin and trypsin) and a combination of non-GI and GI enzymes; (flavourzyme, pancreatin and alcalase) on anti-allergic activity of mung bean protein hydrolysates (MBPH) in RBL-2H3 cell line. In addition, peptide sequences present in the most active mung bean protein hydrolysate were identified using mass spectrometry.

2 | MATERIALS AND METHODS

2.1 | Materials and reagents

Dehulled mung bean seeds were purchased from a local market in Songkhla, Hat Yai, Thailand. Rat basophilic leukemia RBL-2H3 cells were purchased from American Type Culture Collection (ATCC CRL-2256, VA, USA). Alcalase ≥ 2.4 L from *Bacillus licheniformis*, flavourzyme ≥ 500 U from *Aspergillus oryzae*, pepsin ≥ 250 units/mg solid from porcine gastric mucosa, pancreatin 4 $\times$ USP from porcine pancreas and trypsin porcine pancreas ~90 U/mg solid were purchased from Sigma-Aldrich (Steinheim, Germany). Minimum Essential Medium Eagle media (MEM) and anti-DNP-IgE (Mouse Monoclonal anti-DNP) were purchased from Sigma-Aldrich (Steinheim, Germany). Both fetal bovine serum (FBS) and penicillin/streptomycin solution were purchased from Gibco/Invitrogen (Carlsbad, CA). The antibody (dinitrophenylated bovine serum albumin; DNP-BSA) and all other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany). The 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was from Merck Millipore, while the 24-well and 96-well plates were from Nunc. All chemicals used were of analytical grade.

2.2 | Mung bean protein extract (MBPE)

Mung bean protein was extracted by alkaline and acid precipitation according to the method described by Shaheen et al., (2012) with slight modifications. Briefly, the soaked de-hulled mung bean seeds were homogenized at a ratio of 1:6 (w/v) seed to warm water (50 °C) for 2 min and the mixture adjusted to pH 9.5 with 1 M NaOH and mixed for 1 hr to extract the soluble proteins. The mixture was filtered using cheesecloth and passed through filter

TABLE 1 The optimum enzymatic conditions for hydrolyzing mung bean protein extract (MBPE)

Treatment	Enzyme	E/S	Temp (°C)	pH	Sample name
Non-GI	Alcalase	100:1	50	8.0	MBPHA
	Flavourzyme	100:1	50	8.0	MBPHF
GI	Pancreatin	100:1	37	7.0	MBPHP
	Trypsin	100:1	37	7.0	MBPHT
	Pepsin	100:1	37	2.0	MBPHPS
Non GI+GI	Flavourzyme+pancreatin	(50 + 50): 1	50	8.0	MBPHFP
	Alcalase+pancreatin	(50 + 50): 1	50	8.0	MBPFAP

Note. Non-GI is Non-gastrointestinal protease and GI is gastrointestinal protease.

press equipment. The filtrate was centrifuged at $9,300 \times g$, 4°C for 30 min to remove the insoluble materials as a pellet. The supernatant was precipitated at pH 4.5 by addition of 1 M HCl solution and the precipitate recovered after centrifugation ($9,300 \times g$, 4°C for 30 min). Finally, the precipitate was dispersed in distilled water at a ratio of 1:3 (w/v), adjusted to pH 7.0 with 1 M NaOH solution, and freeze-dried (Christ, Deta2-24LSC, UK). The proximate composition of the freeze-dried mung bean protein powder was determined using the method of the association of official analytical chemists (Horwitz, 2000). The dried precipitate was labeled MBPE and contained protein (85.22%), ash (3.64%), fat (1.47%), moisture (1.36%) and carbohydrate (8.65%).

2.3 | Enzymatic hydrolysis of MBPE using non-GI, GI and mixture of non-GI and GI

Mung bean protein hydrolysis was carried out according to the method of Li, Wan, Le, and Shi (2006), with slight modifications. MBPE (1 g) was digested with selected protease using their respective optimum conditions (Table 1). The mung bean protein hydrolysate (MBPH) was taken at intervals of 30 min for 8 hr. The activity of enzyme was stopped by immersing the reaction vessel in a water bath at 95°C for 15 min. After cooling to room temperature, the supernatant containing the hydrolysate was obtained after centrifugation ($10,000 \times g$, 4°C , 20 min). The degree of hydrolysis (DH) was determined following the methods of Nielsen, Petersen, and Dambmann (2001).

2.4 | Preparation of MBPH for anti-allergic assay

The supernatant consisting of MBPHs was filtered through Minisart Syring filter $0.22 \mu\text{M}$ (Sartorius stadium, Germany) to eliminate the sediment and microorganisms. This was later diluted with MEM containing 15% FBS, penicillin (100 units/mL), and streptomycin (100 unit/mL) to obtain the culture media.

2.5 | Anti-allergy activity assay

2.5.1 | Cell culture

The rat basophilic leukemia cell line (RBL-2H3), was cultured using the supplemented MEM in an incubator at 37°C and 5% CO_2 . Eighty

percent cell confluence was trypsinized in 0.25% trypsin-EDTA and suspended in culture media before use.

2.5.2 | β -hexosaminidase inhibition test

MBPH anti-allergic activity in RBL-2H3 cells was assessed using the β -hexosaminidase inhibition assay (Tewtrakul, Itharat, Thammaratwasik, & Ooraikul, 2008). Briefly, RBL-2H3 cells were dispensed in 24 well plates at a density of 5×10^5 cells/ml/well. Anti-dinitrophenyl-immunoglobulin E (anti-DNP IgE) (50 $\mu\text{g}/\text{ml}$) was added, and incubated overnight (24 hr) at 37°C in 5% CO_2 for sensitization of the cells. Afterwards, the cells were washed with 400 μl of buffer A (119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 25 mM piperazine-N,N'-bis 2-ethane sulfonic acid and 0.1% bovine serum albumin BSA, pH 7.2). The cells were then incubated with 160 μl of buffer A for 10 min at 37°C and 5% CO_2 . β -hexosaminidase inhibition and cytotoxic effect of MBPH obtained from pancreatin hydrolysis at 90 min was determined in a preliminary assay in order to optimize protein concentration. The MBPHs at concentrations of 1 ng/ml, 1 $\mu\text{g}/\text{ml}$, 10 $\mu\text{g}/\text{ml}$, 15 $\mu\text{g}/\text{ml}$, 30 $\mu\text{g}/\text{ml}$, 100 $\mu\text{g}/\text{ml}$ and 20 μl of antigen (DNP-BSA, final concentration 0.1 mg/ml) were added to each well and incubated at 37°C for 20 min to stimulate cell degranulation. The supernatant (50 μl) was transferred into a 96 well plate and 50 μl of substrate (1 mM p -nitrophenyl-N-acetyl- β -D-glucosaminide) in 0.1 M citric buffer was added, followed by incubation at 37°C for 2 hr. The reaction was stopped by adding 200 μl of 0.1 M Na_2CO_3 , pH 10.0. The absorbance was measured using a microplate reader at 405 nm. The inhibition (%) of β -hexosaminidase released by test samples was calculated using the following equation:

$$\text{Inhibition\%} = 1 - \frac{(T - B - N)}{(C - N)} \times 100$$

where: (–) indicates without and (+) indicates with the specified ingredient

C = Control; DNP-BSA (+)

T = Test; DNP-BSA (+), Test sample (+)

B = Blank; DNP-BSA (–), Test sample (+)

N = Normal; DNP-BSA (–), Test sample (–)

2.5.3 | Cell viability assay

MBPHs that were positive for β -hexosaminidase inhibition were selected for cytotoxicity test using the MTT colorimetric method (Tewtrakul & Itharat, 2007; Tewtrakul et al., 2008). Briefly, the supernatant of cell culture from previous section with test samples were removed. MTT solution (200 μ l, 5 mg/ml in phosphate-buffered saline) was added to the wells. After incubation for 4 hr, at 37 °C and 5% CO₂, the supernatants were discarded, and isopropanol containing 0.04 M HCl (200 μ l) was added to dissolve the formazan salts production in the cells. The microplate reader was used to measure the density of formazan at an optical density of 570 nm. Cell viability (%) was calculated using the following equation (Tewtrakul et al., 2008)

$$\% \text{ Viability} = 100 - \frac{(C - T)}{C} \times 100$$

C = Control; DNP-BSA (+)

T = Test; DNP-BSA (+), Test sample (+)

2.6 | Mass spectrometry and peptide sequence identification

Mung bean protein hydrolysates derived from 270 min hydrolysis with the combination of flavourzyme and pancreatin (MBPHFP₂₇₀) exhibited the highest anti-allergic activity, and the peptides sequences were analyzed using mass spectrometry according to a previously reported method (Malomo & Aluko, 2016). Potential amino acid sequences of the peptides were determined from the following mung bean protein primary sequences: mung bean seed albumin, 8S globulin β isoform, 8S globulin α isoform, basic 7S globulin 2-like,

β -conglycinin β chain-like, and legumin B-like (<https://www.uniprot.org/>) using the ExPASy Proteomics Server Findpept tool (Swiss Institute of Bioinformatics).

2.7 | Statistical analysis

All data from preliminary test and active MBPHs on β -hexosaminidase and cell viability were subjected to Duncan's multiple range test. The data from DH and β -hexosaminidase inhibition were compared using *t*-test for significance and data were reported as means $\pm$ SEM (*n* = 3). All data analyses were performed using the SPSS software version 17.0.

3 | RESULTS AND DISCUSSION

3.1 | Effect of MBPH concentration derived from pancreatin hydrolysis on β -hexosaminidase inhibition and cell viability

Results obtained from preliminary data showed that after 90 min (DH = 28.65%) digestion of MBPE with pancreatin (MBPHP), there was a significantly high mineral chelating activity (data not shown). Therefore, the effects of MBPHP were examined at different concentrations on the anti-allergy activity and cell viability of RBL-2H3 cell line. As shown in Figure 1, cell viability significantly decreased as the hydrolysate concentration increased. A significant increase of β -hexosaminidase inhibition occurred at 15 μ g/ml of MBPHP, after which the inhibition decreased as hydrolysates concentration increased. The results indicate that the β -hexosaminidase inhibition and cell viability were affected by MBPHP concentration. Hence, we concluded that 15 μ g/ml was the best concentration that showed the highest β -hexosaminidase inhibition. Also this concentration is potentially safe

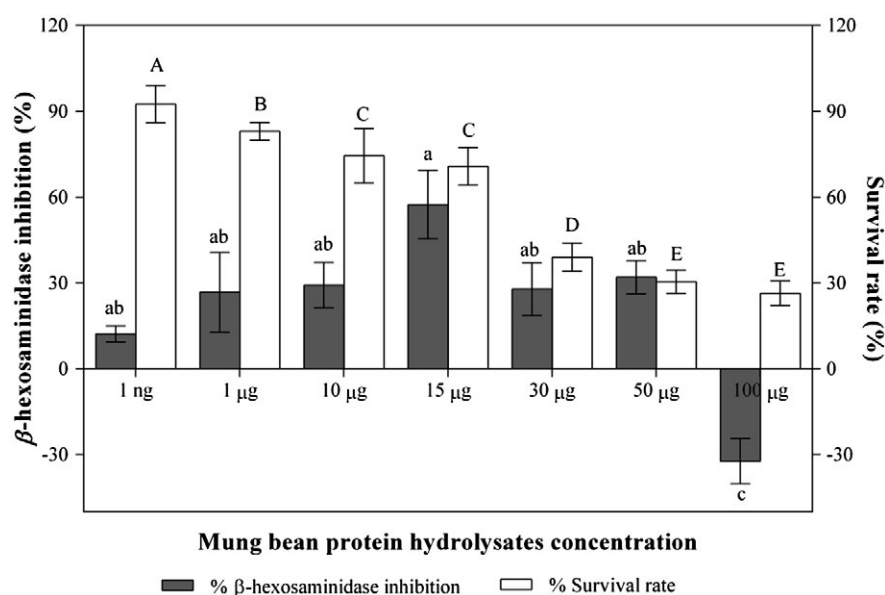


FIGURE 1 Cell viability and inhibition of β -hexosaminidase release after treatment of rat basophilic leukemia RBL-2H3 cells with 90 min pancreatin-hydrolyzed mung bean protein hydrolysate (27.98% degree of hydrolysis)

for use because the cell survival rate was more than 70%. Therefore, 15 $\mu\text{g/ml}$ concentration was then selected for the next study.

3.2 | Effect of MBPHs derived from non-GI enzyme digestion on β -hexosaminidase inhibition

Non-GI enzymes from *Bacillus licheniformis* (alcalase) and *Aspergillus oryzae* (flavourzyme) were used to hydrolyze the MBPE under controlled conditions. From preliminary results, the optimized concentration selected for further study was 15 $\mu\text{g/ml}$, because it showed the highest β -hexosaminidase inhibition with minimal cytotoxic effect. Effect of MBPH from alcalase hydrolysis (MBPHA) on β -hexosaminidase inhibition is shown in Figure 2a. The inhibitions of β -hexosaminidase released using treated samples were separated into two groups. The first group recorded more than 100% inhibition, which occurred at 90, 210, and 240 min hydrolysis (with corresponding DH of 27.98, 37.52 and 39.83%, respectively) when

compared with control. The results are similar to those reported for hypoallergenic chickpea hydrolysate obtained from 180 min alcalase hydrolysis with 27% DH (Clemente et al., 1999). Moreover, hydrolysis with alcalase was more effective in reducing lentil allergen, because the lentil hydrolysates (180 min, 24% DH) inhibited 88% of specific IgE-binding allergens (Cabanillas et al., 2010). The major soybean allergens (Gly m Bd 30 K, Gly b Bd 28 K and subunit of β -conglycinin) were also degraded using *Bacillus* sp. protease hydrolysis for 30 min with 25% DH (Tsumura et al., 1999). In addition, alcalase assisted hydrolysis reduced buckwheat, peanut and soymeal allergens (Cabanillas et al., 2012; Sung, Lee, et al., 2014; Wang et al., 2014). Ashaolu and Yupanqui (2017) found that soybean protein hydrolysate prepared using alcalase digestion can inhibit β -hexosaminidase released during RBL-2H3 cells degranulation. Alcalase is an endopeptidase, which destroys the cross-linking ability of an allergen and the cleavage of this cross link may be sufficient to eliminate allergenic activity (Clemente et al., 1999).

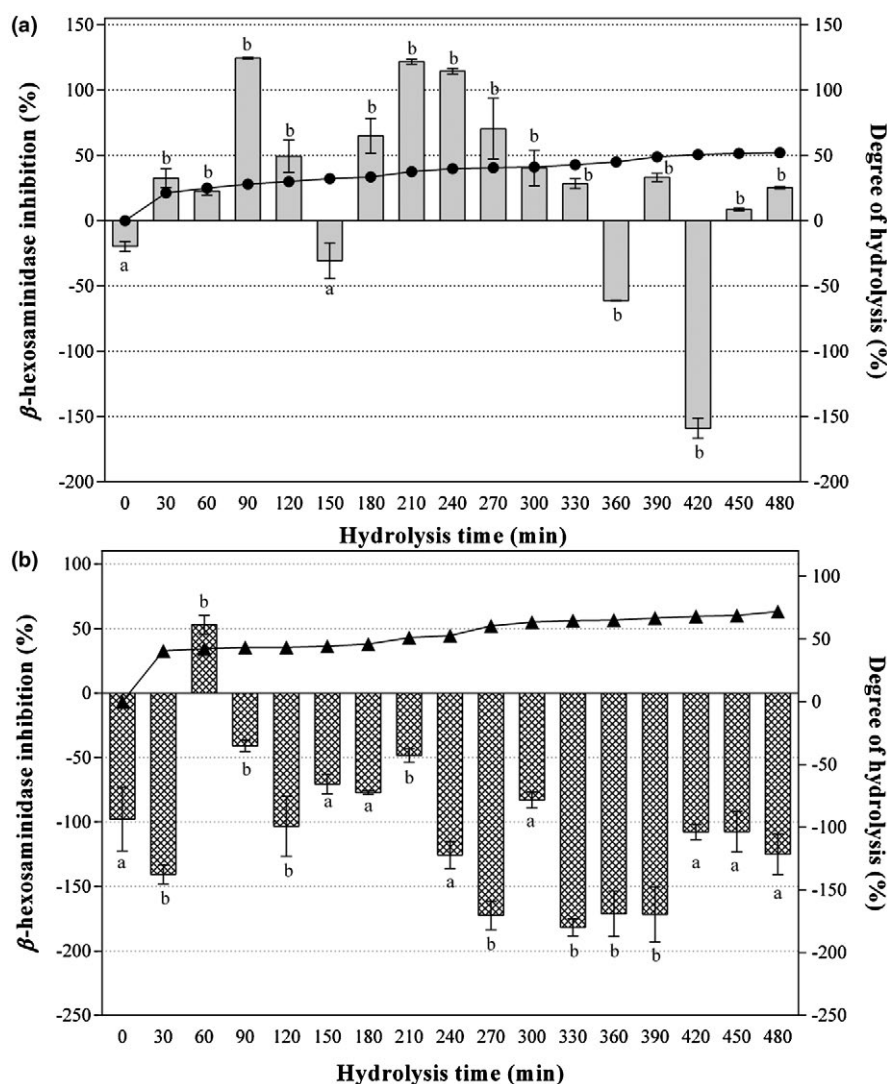


FIGURE 2 Effect of mung bean protein hydrolysis on β -hexosaminidase secretion from antigen stimulated RBL-2H3 cells. The significant differences between optimum condition group (0 min) and 16 experimental groups were determined by the student's t test ($p < 0.05$) for alcalase (a) and flavourzyme (b)

The second group showed the β -hexosaminidase inhibition range from 8.65%–70.36% without any relationship with DH and hydrolysis time. MBPHA₁₅₀ inhibited β -hexosaminidase production after hydrolysis treatment for 150 min (33.41% DH) when compared with the control. Moreover, MBPHA₃₆₀ and 420 (DH 45.04% and 50.65%) significantly ($p < 0.05$) promoted the β -hexosaminidase production. Thus, alcalase might cleave protein and increase target allergens for β -hexosaminidase release during RBL-2H3 cells degranulation. The variations in the effectiveness of the hydrolysates based on their DH can be attributed to the peptides obtained at the different hydrolysis time and the mechanism of action of the peptides on the RBL-2H3 cells to induce or inhibit generation of β -hexosaminidase.

The effect of MBPH prepared with flavourzyme (MBPHF) on β -hexosaminidase inhibition is shown in Figure 2b. MBPHF₆₀ showed significant ($p < 0.05$) 53% inhibition of β -hexosaminidase at 42.30% DH when compared to the control. This might be due to the partial hydrolysis of MBPE and ability of flavourzyme to destroy highly active surface epitopes of the allergen after 60 min hydrolysis. Our result is on par with a previous study by Clemente et al. (1999), who reported that FCPH₁₈₀ showed inhibition at 20 and 50% of antigenic activity. On the contrary, MBPHF₉₀₋₄₈₀ increased anti-allergic activity when compared to the control. The DH and hydrolysis time of MBPHF did not affect anti-allergy activity. There are many studies in which flavourzyme hydrolysis was employed, with increased the observed allergenic activity. These include roasted peanut (Cabanillas et al., 2012), chickpea (Clemente et al., 1999) and buckwheat (Sung, Lee, et al., 2014). The majority of legume seed allergens are presumably comprised of conformational epitopes, which are highly heat-labile, susceptible to enzymatic degradation, and difficult to isolate, often making standardized extracts for diagnostic purpose unsatisfactory (Sampson, 2004). Conformational epitopes are destroyed when the native protein shape is altered by hydrolysis whereas sequential epitopes are not affected (Sampson, 2004). As noted earlier, flavourzyme can act as an endopeptidase and an exopeptidase or aminopeptidase, in order to cleave proteins to produce small peptide chains and free amino acids (Sung, Lee, et al., 2014). As a protease, flavourzyme might have induced the exposure of the interior hidden epitopes of the allergenic proteins. The MBPHF may also promote the interaction of IgE with immune system in our body and lead to a potential increase in protein allergenicity (Nowak-Węgrzyn & Fioocchi, 2009).

3.3 | Effect of MBPH using GI digestion on β -hexosaminidase inhibition

The GI proteases used for MBPH production include pancreatin (MBPHP), trypsin (MBPHT) and pepsin (MBPHPS). MBPHP₉₀ showed significant ($p < 0.05$, DH = 28.65%) 80.32% inhibition of β -hexosaminidase released when compared to the control (Figure 3a). In addition, MBPHP_{60, 330 and 450} also had inhibitory effects on β -hexosaminidase released in RBL-2H3 cells, except that samples had lower than 80% inhibition. The results obtained are similar to a previous work whereby allergens in defatted soybean flour were digested using pancreatin for 360 min to obtain 23.5% DH (Barca,

Wall, & López-Díaz, 2005). The hydrolysates, which were fractionated using 1–10 kDa membrane ultrafiltration increased anti-allergy activity (Barca et al., 2005). Lee et al. (2007) later confirmed that pepsin and chymotrypsin reduced 11S globulin allergen after hydrolysis for 120 min. Ara h 1 and Ara h 2 of peanut allergens were also degraded by trypsin and chymotrypsin hydrolysis for 60–180 min (Yu et al., 2011). However, MBPHP showed allergic activity in partial hydrolysis as evident in Figure 3a. Pancreatin is a mixture of endo- and exo- peptidases, and may help to increase stability of peptide epitopes during hydrolysis time. This study indicates that DH was minimal with trypsin over 480 min (Figure 3b). Eight of 17 hydrolysates samples from MBPHT showed significant increase in allergic activity when compared to control. Legume allergens are stable in the gastrointestinal fluid, which contains pepsin, trypsin pancreatin, and chymotrypsin (Verma, Kumar, Das, & Dwivedi, 2013). It has been reported that α , α , and β -subunits of β -conglycinin in soybean allergen resisted 10 min trypsin hydrolysis (Wang et al., 2014). Also, tryptic hydrolysis of soy bean protein isolate did not reduce its allergic activity (Panda, Tetteh, Pramod, & Goodman, 2015). None of MBPHT showed β -hexosaminidase chelation possibly due to inability of trypsin to sufficiently expose active epitopes to inhibit IgE chelation.

The MBPE was stable to pepsin digestion until 480 min hydrolysis because of low DH as shown in Figure 3c. However, only MBPHPS₂₇₀ showed β -hexosaminidase inhibition while other MBPHPS samples induced allergic activities. Several works have investigated the anti-allergic effect of pepsin digestion in legume allergens. Sormus de Castro Pinto et al. (2009) reported that sweet lupin, chickpea and lentil 11S globulin allergens were reduced after hydrolysis with pepsin for 60 min. Soybean 2S protein allergen was partially reduced using peptic hydrolysis for 90 min (Sung, Ahn, et al., 2014). The β -hexosaminidase release in RBL-2H3 cell degranulation significantly decreased after peptic hydrolysis of soybean protein (Ashaolu & Takahashi Yupanqui, 2017). On the other hand, some legume allergens exhibit very high stability to peptic digestion. For example, soybean β -conglycinin was not destroyed after 60 min of pepsin hydrolysis (Astwood et al., 1996; Zhao et al., 2010). Ara h 1 is a major allergen found in peanut; in vitro gastric digestion led to rapid breakdown into smaller fragments, but still retained its allergenicity to induce histamine release in basophils (Eiwegger et al., 2006). The results obtained in this experiment confirm that legume seed allergenicity may not be reduced by peptic hydrolysis as mentioned previously. Moreover, 8S globulin in mung bean is a high molecular weight (150 kDa) homologue (Itoh et al., 2006), which is stable to peptic digestion. Pepsin is a site-specific enzyme with preference for peptide bonds formed by certain amino acids (Whitaker et al., 2003); therefore, it may have limited ability to split or destroy all epitopes on an allergenic protein.

3.4 | Effect of MBPH using combination of non-GI and GI digestion on β -hexosaminidase inhibition

MBPHF₂₇₀ at 97.44% DH showed significant ($p < 0.05$) inhibition of β -hexosaminase released (135.61%) in RBL-2H3 cells (Figure 4a).

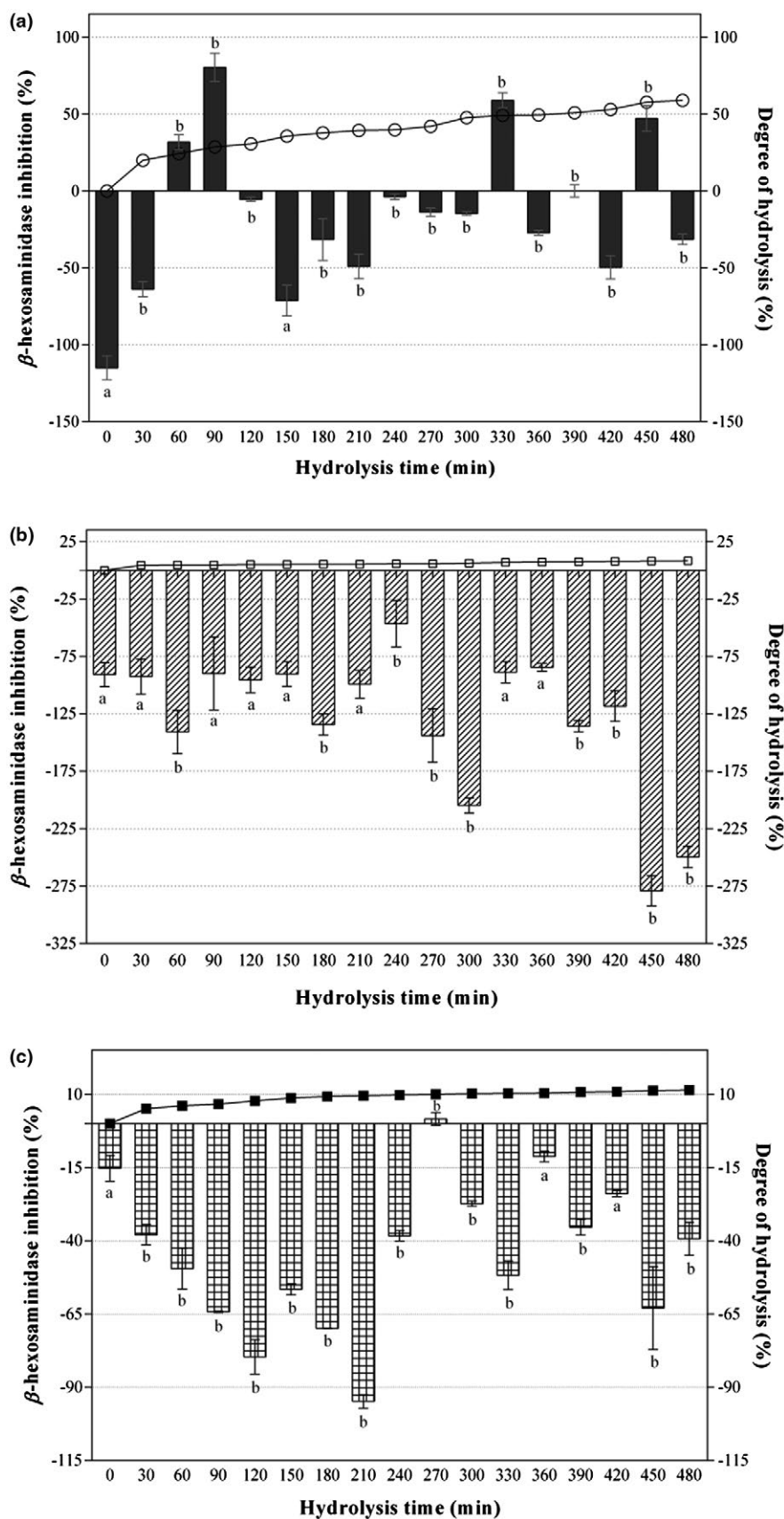


FIGURE 3 Effect of mung bean protein hydrolysis on β -hexosaminidase secretion from antigen stimulated RBL-2H3 cells. The significant differences between optimum condition group (0 min) and 16 experimental group were determined by the student's *t* test ($p < 0.05$) for pancreatin (a), trypsin (b), and pepsin (c)

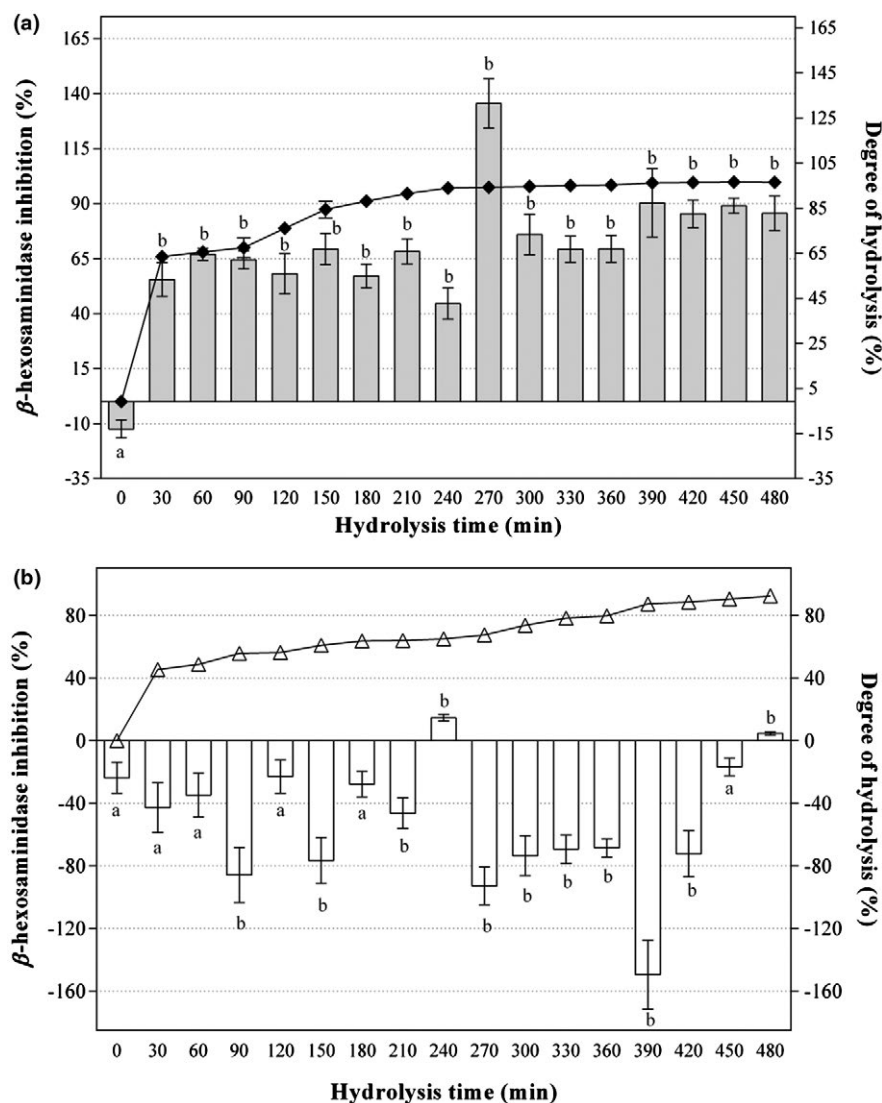


FIGURE 4 Effect of mung bean protein hydrolysis on β -hexosaminidase secretion from antigen stimulated RBL-2H3 cells. The significant differences between optimum condition group (0 min) and 16 experimental group were determined by the student's *t* test ($p < 0.05$) for combination of flavourzyme + pancreatin (a) and alcalase + pancreatin (b)

All of MBPHFP samples effectively induced β -hexosaminidase inhibition compared to the control. However, MBPHFP_{270, 390-480} at DH of 97.44 and 99.38–99.76%, respectively were used for further study because they showed 80% β -hexosaminidase inhibition. Our finding agree with that of Clemente et al. (1999) who hydrolyzed chickpea 11S globulin with alcalase and flavourzyme, sequentially. They found that the hydrolysates with DH >34% decreased the antigenicity activity by >90%. The sequential treatment with alcalase and flavourzyme hydrolysis was applied to reduce allergens in various legumes such as lentil (Len c 1, c 2, 56% DH) (Cabanillas et al., 2010), kidney bean, black gram and peanut (DH of 48.4%, 63.5%, 69.4%, respectively) (Kasera et al., 2015)). Flavourzyme and pancreatin are peptidases which when combined may confer anti-allergic effect on hydrolysates directly in relation to DH. They might aid in the destruction of mung bean protein allergens into smaller peptides or decrease a number of hidden epitopes.

The MBPHAP₂₄₀ and MBPHAP₄₈₀ at DH of 64.97 and 92.34% partially showed a reduction of β -hexosaminidase release in RBL-2H3 cells at 14.60 and 4.62%, respectively (Figure 4b). This work clearly indicate that DH and hydrolysis time were not related to anti-allergic activity. Moreover, 9 of 17 hydrolysates treatments significantly inhibited β -hexosaminidase release while other hydrolysates were similar to the control. Alcalase is classified as a serine peptidase (EC 3.4.21.62), particularly subtilisin-like type, and acts as an endopeptidase that produces high yield of peptides (Sung, Lee, et al., 2014). Alcalase was shown to have degraded allergenic proteins to produce low molecular weight peptides below 10 kDa, and showed the most effective result in reducing allergenicity (Sunng, Lee et al., 2014; Wang et al., 2014; Pessato et al., 2016). Pancreatin consists of endoexopeptidases such as trypsin, chymotrypsin, elastase, carboxypeptidase-A and carboxypeptidase-B (Whitcomb & Lowe, 2007). Thus, the rationale for the combination of alcalase and pancreatin enzymes

is to obtain maximal hydrolysis that will generate peptides with reduced allergenicity. Our findings indicate that the combination of endo-peptidases (alcalase) and endo and exo peptidases (pancreatin) produced an initial digest with hypoallergenic peptides.

This work indicate that β -hexosaminidase production can be affected by enzyme type. The combination of flavourzyme and pancreatin hydrolysis was the most effective on β -hexosaminidase inhibition followed by alcalase and pancreatin, respectively (Figure 5). MBPHFP₂₇₀, MBPHA₉₀ and MBPHP₉₀ significantly (Zhao et al., 2010) reduced allergic activity when compared to other groups. The effects of MBPHFP, MBPHA and MBPHP on cell viability were determined using the MTT assay (Figure 6). The hydrolysates had minimal toxicity effect on RBL-2H3 cells because all the samples showed more than 70% cell viability. Thus, it can be summarized that concentration of MBPH had effect on β -hexosaminidase inhibition and cell viability. MBPHFP exhibited the most positive anti-allergic activity and minimal toxicity in RBL-2H3 cells. The DH and time of hydrolysis were minor factors considered for producing anti-allergy peptides. MBPHFP and other active hydrolysates may be used to substitute food formulation ingredients as protein based and hypoallergenic ingredients.

3.5 | Anti-allergic peptide sequences

MBPHFP₂₇₀, showing the highest anti-allergic activity was analyzed on a Q-TRAP mass spectrometry. According to the results shown in Figure 7, most of the ions were within the range of 103.4–1,935.3 Da. The observed mass of MBPHFP₂₇₀ obtained from MS analysis was in the range of 191.2–1884.2 Da. Some researchers have also reported

low (<10 kDa) MW hydrolysates derived from various source such as soybean hydrolysate (0.5–3 kDa) (Barca et al., 2005), acid bovine β -lactoglobulin hydrolysate (1.63 kDa) (Prioult, Pecquet, & Fliss, 2005) and soybean protein hydrolysate (1–10 kDa) (Barca et al., 2005). The results indicate that MBPHFP₂₇₀ with low MW peptide sequences affect mast cell stability by blocking IgE-allergen binding (insufficient number of epitopes) that have functional role in degranulation of mast cells. Moreover, peptides with low MW may reduce the aggregation of IgE-peptide adjacent receptor on mast cell. The low MW peptides may easily attach onto cytoplasmic membrane of mast cell and create strong boundary wall to prevent mast cell degranulation. However, high MW (>10 kDa) hydrolysates also decreased the allergic activity. The β -conglycinin allergen from soybean protein was reduced by alcalase and trypsin hydrolysis according to ELISA results of soybean hydrolysates (21.0 and $\leq$ 14.4 kDa) (Wang et al., 2014). In addition, high MW (7–16 kDa) whey protein isolate hydrolysate also decreased the allergic detection (Pessato et al., 2016). In the presence of peptides with large MW, β -hexosaminidase enzyme may be unable to release the cell membrane and degranulate. However, the large MW peptides still contained higher level of allergenic fragments than smaller peptides. This finding might be summed up by suggesting that low MW peptide sequences such as the di-, tri-, tetra-, penta-, and other peptides in MBPHFP₂₇₀ interacted synergistically to inhibit IgE-binding and mast cell receptor during cell degranulation. Thus, the decreasing of allergic activity could be due to an increase in number of small MW peptide resulting from breaking down of allergen epitopes of mung bean protein as indicated by the observed masses in Table 2. The data suggest that peptide sequences in MBPHFP₂₇₀ were abundant in low MW peptides (191.2–1884.2 Da) with the potential of increasing their

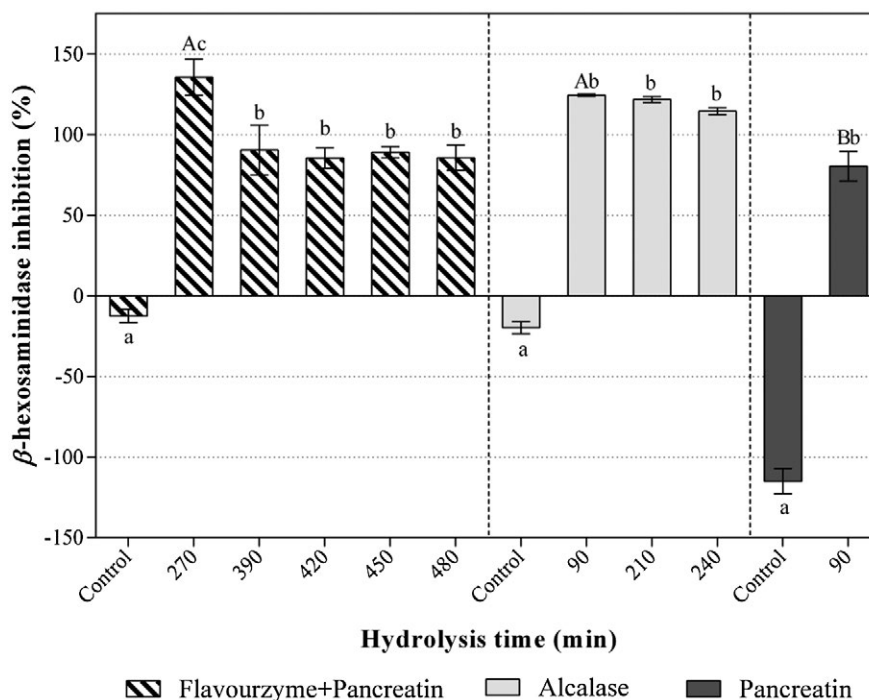


FIGURE 5 Inhibition of β -hexosaminidase release by mung bean protein hydrolysates obtained after protein hydrolysis with flavourzyme +pancreatin, alcalase or pancreatin

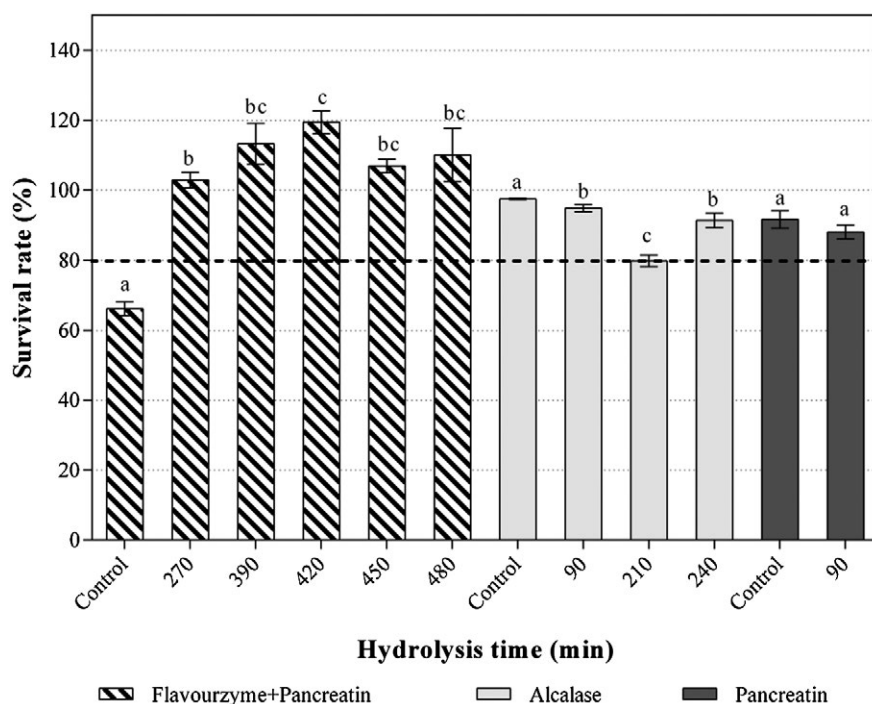


FIGURE 6 Viability rate of rat basophilic leukemia RBL-2H3 cells 24 treated with 15 µg/mL mung bean protein hydrolysates (MBPHs) obtained from flavourzyme + pancreatin, alcalase, and pancreatin hydrolysis

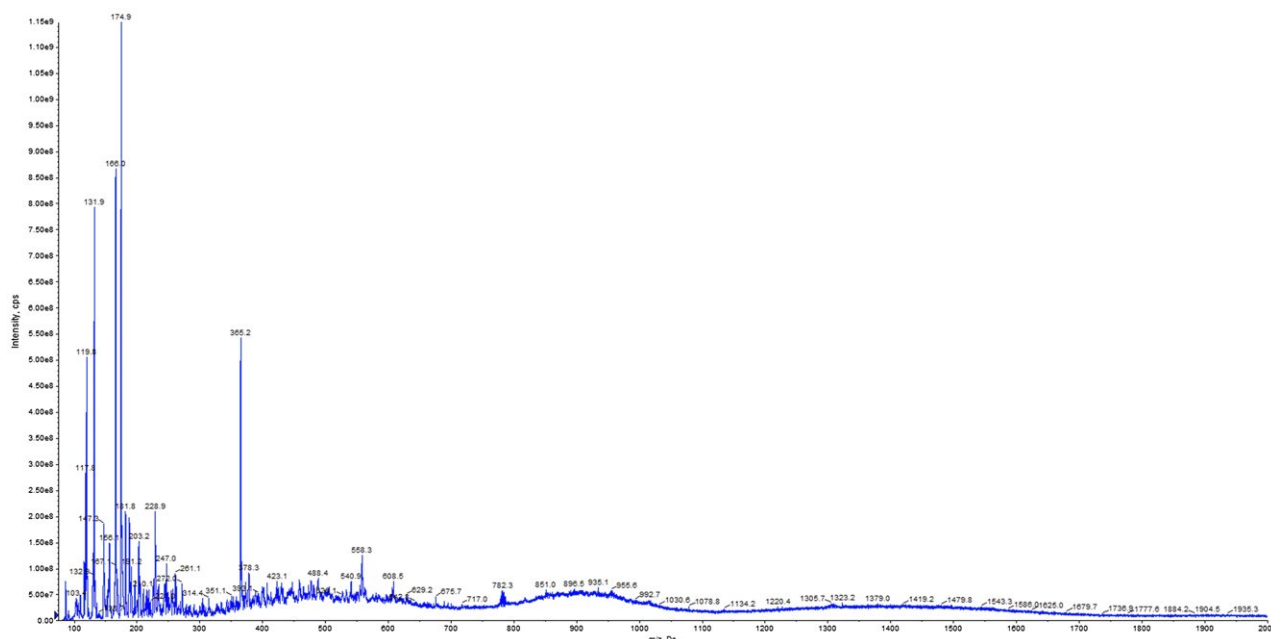


FIGURE 7 Mass spectrum of the mung bean hydrolysate obtained from protein hydrolysis with flavourzyme + pancreatin

anti-allergy activity. The peptide sequences of MBPHFP₂₇₀ are presented in Table 2 showing a total of 226 different identified peptides. The amounts of peptide sequences in MBPHFP₂₇₀ are huge because it consists of a mixture of peptides with different sizes. The analysis of peptide sequences in MBPH₂₇₀ showed a high amount of low

molecular weight <1 kDa, containing 150 of 226 peptide sequences was represented in Table 2. Tripeptides were the most abundant and represented 39 of 226 peptide sequences followed by tetrapeptides (37 peptide sequences) and pentapeptides (27 peptide sequences). The peptides contained hydrophobic amino acids such as proline (P),

TABLE 2 Potential peptides in the hydrolysate derived from 270 min hydrolysis of mung bean protein extract by a mixture of flavourzyme and pancreatin

Parent protein	Observed mass (Da)	Potential peptides
Mung bean seed albumin,	191.2	AT, TA
Legumin B-like,	203.2	PS, SP, AI, AL, LA, IA
β -conglycinin, β -chain like,	221.0	SD, DS, VC, MA, CV
8S globulin α isoform, 8S	315.2	MSE,AQP,PAQ,VTF, KAP
globulin β isoform and	351.1	NSM, GQF, QGF, QFG, MSN
basic 7S globulin 2-like	366.2	FAE, PSY, SYP, PYS, AYI,TVF, AYL, FIS, FLS, YIA, SIF, IFS, LPH, HPL
	378.3	YPV, VYP, IVF, VFL, FLV, VLF, VFI
	393.1	RCD, GETS, GGPY, DMK, SSLS, LSSS, SSSL, STSV, SLSS
	407.2	DGDT, GDTD, TEAS, STEA, SSVD, GFPS, YAPG,SGFP, LFK, TWT, WTT, MKE, SSTL, LSST, TLSS, GFAL, AFGI, FLAG, LFK, KLF, KFL
	423.1	SSTE
	488.4	DKIL, LVGSI, VKLE, VEKL, IKLD, VGLSL, LKDL, VELK, RLIS, SLIR, GKKG, KVNK, NKKV, RGKK, KGRK
	558.3	HGEES, SSCVY, VNPND, QFTY, NPDGR, HFTPG, IDLTP, PTVEL, PTEVL, IPTEV, ISEIP, LNKPS, LAVNAA, NKPSL, LQQVA, QQVAL, VLNGR, DLVIV, LLSLL, ILTLV
	629.2	EHQES, QPEQQ, PEQQQ
	642.3	GSDYNS, EAYLF, AEYFI, SLHNAT, PSLAGTP, PGSPAT, VGDVRP, LLPHY, RNQKP, WPVLK
	782.3	DFGSIED, QNNPFY, CFNSRR, LEACFLS, LEQGHQAQ, KNNPFY
	896.5	VIGGYQME, PDGRDSHI, LTKDFGS, TEKDFGSI, IDSAFRST, RIDYDSK,VLKAFCLC, GVEWRIH, VEWRIHG, HLVPHFF, PILDGWPV, KFGVEGFL, NLHVPAFV, INHLSLVT, NHLSLVTI, HLSLVTIN, IRELTKH, VPAIDLVLG
	955.6	EGHKGRKGS, PIEKDPRT, VRPILDGW, SRRVGNVPA, EAKIELVGP, AILTLVNP, RIHFTPGK, KNPQLKDL, MVRARIPL, ILTNRLI, LLGLARGKK, SKAIVLVI
	969.6	EVYLFKGN, AGTTFVLN, LFKGDYV, KYVRLQY, HKGRKGSLS, FGHRLVLQ, QFGHLRVL, TLVGDRPI, IVILVINEG
	1,078.6	FNSDRRFH, SVDMEKGSLL, EQGHAQKIPA, IGVIGGFSTD, ELIYARGSG, AGTIFFLVN, PAGTIFFLVN, LAVNAASKPH, FLGETSKALI, GTKLSTVIPP, PQLHNTKKL, IKLAIPVNNP, ARIPLLLLLG
	1,305.7	QCLGFVDAGLEPG, FRFSSRDYEV, HVPFVSSCVYP, PVLNGTGFESEID, LAGEKDNVMSEI, LSSQDEPFNL, SSSSLAVNAASKP, TPKKAEALFEGD, DDVFVIPAAYPV, KQIQNLNRY, LRNSNPIYSNK, LQRFDRSKQ, DVFIIPATYPVA, RLISGFPPLAGT, VELIYARGSGK, YIARGSGKIEIV, LSLVTINKAFIS, SPIATSIVIGGYQ, RFDQRSKQIQ
	1,478.8	NSDRWFHTLFR, SDRWFHTLFRN, EDDVFIIPATYPV, PIATSIVIGGYQME, LEVQRYRAEVSE, PVAINATSNLFFA, GWPVLKAFCLCEL, QKDLDFISSVD, DSDIKEISRVLF, FPVLRNTVFADSI, QRKFAICLPSTSK, RRVGNVPAIDLVLG
	1625.0	LFKNQYGHRLVIH, KPSLSCINHLSLVTI, GSLLPHYNSKAIVI, VIVELKREQUIRE
	1679.7	DAQPEQQQREEGHK, PLKTGCTNNTCGVQPF
	1736.9	EFDLASSRFGFSSSL, IFSSTLQQVALNVDSD, DSDIKEISRVLFGE, QVALNVDSDFKQLFI, LTGAIGVIGGFSTDLSK, TTRARAPSNLHVPAF, NSRRVGNVPAIDLVLGG
	1884.2	GVEGFLGLARGKKGVGL

alanine (A), phenylalanine (F), glycine (G), leucine (L), and isoleucine (I); however, hydrophilic aspartic acid (D), threonine (T), glutamic acid (E), glutamine (Q), arginine (R), histidine (H), serine (S) and lysine (K) were also present. In the present results, peptides with highly hydrophobic and hydrophilic amino acids may easily bind to the ϵ heavy chain of IgE via antigen binding region; this causes the blocking of IgE and Fc ϵ R1 on mast cells (Agadagba, 2014). The receptor on mast cell might be inactivated via hydrophobic/hydrophilic reaction of peptides with

tetrameric receptor complex. Several studies reported that hydrophobic and hydrophilic amino acid residues in hydrolysates affect anti-allergic activity. Amino acid pattern of soybean protein hydrolysate fraction between 0.5–3 kDa was rich in F + Y, L, K, I and T residues; in addition, it was potentially useful for hypoallergic formulas (Barca et al., 2005). The whey protein isolate hydrolysed with alcalase comprised larger size and more hydrophobic peptides that could partially inhibit IgE binding capacity (Pessato et al., 2016). Ashaolu &

Takahashi Yupanqui (2017) reported that soybean protein hydrolysates that contain the most abundant hydrophobic amino acid residues (L, I, K, F,) and hydrophilic amino acids (E, D, and R) also have potential anti-allergic activity property. These findings indicate that hydrophobicity and hydrophilicity of amino acids in protein hydrolysates also influence anti-allergic activity. Moreover, amino acid position and peptide sequence also play important roles in anti-allergic activity. Several researchers reported that peptides obtained from various sources of protein hydrolysates with different sizes, residues and sequences affect the anti-allergic activity of products such as egg white, soybean and/or synthetic peptides (Buku, Condie, Price, & Mezei, 2005; Kim, Kim, Kim, Ro, & Jeoung, 2008). Moreover, the substituted amino acid residues of synthetic peptides influenced the inhibition of histamine release in RBL cells line. In the present work, abundance of peptide sequences with different size, length, and amino acid residues was found in the MBPHFP₂₇₀ hydrolysates. As mentioned earlier, the low MW and amino acid residues in peptide sequences may be strong inhibitors of IgE-binding or IgE/receptor interaction on the mast cell. Therefore, the sequence, structural, conformation and amino acid position also play an important role in anti-allergic activity of peptides via IgE/receptor interaction inhibition. For example, the 2,587.2 Da mast cell degranulation peptide (MCD: IKCNCKRHHVHKHICRICKGKN) may facilitate the design of analogues to inhibit IgE binding (Buku, Price, Mendlowitz, & Masur, 2001). This peptide provides direct evidence of binding to RBL cells through a membrane receptor followed by endocytosis (Buku et al., 2001). Endocytosis is a process important for the functioning and recycling of all membrane receptors (Buku et al., 2001). The peptide with cyclic disulfide structure influenced inhibition of IgE binding such as the one that involves the MCD peptide (Buku, Mendlowitz, Condie, & Price, 2003). In 4 of the 226 peptide sequences, C residues with a sulfhydryl side chain that can promote disulfide formation peptide in a peptide such as VLKAFCLC (896.5 Da) may have strong inhibitory activity against IgE or prevent receptor-ligand binding. Moreover, cyclic peptides such as SSCVY (558.3 Da), CFNSRR (782.3 Da), and PLKTGCTNNTCGVQPF (1679.7 Da) may appear via a connection between the singlet SH-group and carboxyl ends of the peptide. In addition, some of MBPHFP₂₇₀ peptides had N-terminal isoleucine (I¹), which is similar to MCD peptides, including I¹LTLV (488.4 Da), I¹LTLV, I¹PTEV, I¹SEIP (558.3 Da), I¹DSAFRST, I¹NHLSLV, I¹RELTKH (896.5 Da), I¹LTLNRLI (955.6 Da), I¹VILVINEG (969.6 Da), I¹GVIGGFSTDL, and I¹KLAIPVNNP (1,078.6 Da). The results suggest that conformational structure and position of C and I¹ residues influenced the reduction of β -hexosaminidase release and/or inhibition of IgE/receptor binding. IKCNCKRHHVHKHICRICKGKN, an MCD (2,561.3 Da) synthetic peptide analog showed inhibition of β -hexosaminidase enzyme in mast cells. Buku et al., (2005) described that the A¹² substitutions influence the primary side chain peptide during IgE-receptor interactions. The GVEGFLLGLARGKKGVLLGL (1884.2 Da) in MBPHFP₂₇₀ had almost similar peptide size and hydrophobic amino acids such as G, V, A to [A¹²] MCD peptide. Moreover, the V⁶ and A¹² MCD substitutions proved to be an excellent inhibitor of IgE-mediated mast cell degranulation, based on the result of the β -hexosaminidase

assay (Buku, Keselman, Lupyan, Mezei, & Price, 2008). Structural changes arising from V⁶ and A¹² MCD peptide analogs led to high in helical (α -helix) content, which was found to enhance the inhibitory effect of the peptides. This is because most of the C-terminal region becomes structurally conserved as an α -helix. Besides the N-terminal, the relationship between C- and N-terminals is important for receptor recognition binding in MCD peptide (Buku et al., 2008). The V⁶ and A¹² MCD, with their hydrophobic amino ends may interact through hydrophobic interactions with tryptophan residues in D2 of receptor in such a way as to produce maximum inhibition (Buku et al., 2008).

As mentioned above, the V⁶ and A¹² MCD peptide proved to a strong inhibitor of β -hexosaminidase release in RBL cells line. The activity of peptide sequences in MBPHFP₂₇₀ with V and A residues may be attributed to diverse binding of peptides to one or the other domain (D1 and 2) of the receptor. For example, penta- and hexa-peptides; LQQVA, QQVAL and LAVNAA (558.3 Da), octa- and nona-peptides; VLKAFCLC, NLHVPAFV, VPAIDLVLG (896.5 Da), MYRARIPL, EAKIELVGP, AILTLVNP, SRRVGNVPA, SKAIVILVI (955.6 Da), AGTFFFLVN (969.6 Da), AGTIFFLVN (1,078.6 Da), deca-peptides; PAGTIFFLVN, LAVNAAASKPH, IKLAIPVNNP (1,078.6 Da), and others; QCLGFVDAGLEPG, HVPFVSSCVYP, LAGEKDNVMSEI, SSSSLAVNAAASKP, VELIYIARGSGK, YIARGSGKIEIV, LSLVTINKAFIS, DVFIIPATYPVA, DDVFVIPAAYPV, SPIATSIIVIGGYQ (1,305.7 Da), EDDVFIIPATYPV, PIATSIIVIGGYQME, LEVQRYRAEVSE, PVAINATSNLNFFA, GWPVLKAFCLCEL (1,478.8 Da). These peptides may interact via hydrophobic interaction with domain D1 or 2 of receptor. Kim et al., (2008) studied the effect of peptides on histamine release from passively sensitized lung mast cells by ovalbumin. Results indicated that LVAHVGAGGVL, LSYLLWRSRLP, TDGVTYTNDCI, RVSSCRGRNHIV, PHVMRGGEYSGA and ETIGARWVRIE inhibited histamine release from lung mast cells when compared with the control ovalbumin-treated group. These peptides contained 11–12 residues, which had hydrophobic (L, V, A, G, Y, W, P, I, C, M), and hydrophilic (D, E, H, N, S, T) amino acids. As above mentioned, all these amino acids and peptides (11–12 amino acid residues) corresponding to IGVIGGFSTDL (1,078.6 Da), HVPFVSSCVYP, LAGEKDNVMSEI, LSSQDE PFNLR, TPKKAEALFEGD, DDVFVIPAAYPV, LRNSNPIYSNK, DVFIIPATYPVA, VELIYIARGSGK, YIARGSGKIEIV, LSLVTINKAFIS (1,305.7 Da), and NSDRWFHTLFR, SDRWFHTLFRN, LEVQRYRAEVSE (1,478.8 Da) were present in the MBPHFP₂₇₀.

Therefore, the binding of multivalent MBPHFP₂₇₀ peptides may indicated that low MW (<10 kDa) peptides could easily interact or attach to IgE, receptor or IgE-receptor interaction via hydrophobic bonding to inhibit their active domain. In addition, all peptide sequences may be able to interact with the membrane receptor and possess cell penetration ability as have been shown for some transcription factors (Buku et al., 2001; Lindgren, Hällbrink, Prochiantz, & Langel, 2000). These small peptides in MBPHFP₂₇₀ could reduce the interaction of IgE with the receptor (Helm, Spivey, & Padlan, 1997), which may then lead to reduction of β -hexosaminidase release. Moreover, the formation intra-peptide disulfide bonds may have produced peptide structures in MBPHFP₂₇₀ that inhibited

IgE-binding and receptor interaction. The physiochemical structure of peptides may have also been an important factor for the observed anti-allergic activity. This is because peptides with high C-terminal α -helix conformation have been shown to enhance the inhibition of β -hexosaminidase release and IgE-receptor interactions. Our current finding with the MBPHFP₂₇₀ peptides may fit these criteria based on the location of specific amino acid residues that have been shown to promote α -helix conformation. Thus, the effect of combined use of GI and non-GI enzymes, degree of hydrolysis, amino acid residues, and peptide sequences were the most important factors to reduce mung bean protein allergenicity.

4 | CONCLUSION

Soluble mung bean protein allergens were directly affected by protease type, DH and hydrolysis time. The combination of flavourzyme and pancreatin had the highest anti-allergic activity effect using β -hexosaminidase inhibition during RBL-2H3 cells degranulation and minimal toxicity. In addition, MBPHs prepared with individual alcalase and pancreatin hydrolysis also inhibited β -hexosaminidase release. All MBPHs prepared with flavourzyme and pancreatin, as well as alcalase and pancreatin, had minimal toxic effect on the RBL-2H3 cells. The MBPHFP₂₇₀ peptides identified comprised 226 difference peptides with most of them in the range of 191.2–1884.2 Da. The MBPHFP₂₇₀ peptides contained hydrophobic (P, A, F, G, L, I, W, Y) that may have influenced inhibition of β -hexosaminidase release during RBL-2H3 cells degranulation. Thus, the mung bean protein hydrolysates can be useful as substitutes in hypoallergenic food formulations, for population groups with specific nutritional requirements.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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REFERENCES

- Abbas, M., & Shah, H. (2007). Proximate and mineral composition of mung bean. *Sarhad Journal of Agriculture*, 23, 463–466.
- Agadagba, S. K. (2014). Therapeutic antibody case study: Humanized xolair. *Advances in Life Sciences*, 4, 114–118. <https://doi.org/10.5923/j.als.20140403.03>
- Ashaolu, T. J., & Takahashi Yupanqui, C. (2017). (Ashaolu, 2017 #415). Suppressing activity of enzymatically-educed soy protein hydrolysates on degranulation in IgE-antigen complex-stimulated RBL-2H3 cells. *Functional Foods in Health and Disease*, 7, 545–561. <http://doi.org/10.31989/ffhd.v7i7.35>
- Astwood, J. D., Leach, J. N., & Fuchs, R. L. (1996). Stability of food allergens to digestion in vitro. *Nature Biotechnology*, 14, 1269. <https://doi.org/10.1038/nbt1096-1269>
- Barca, A. M. C. D. L., Wall, A., & López-Díaz, J. A. (2005). Allergenicity, trypsin inhibitor activity and nutritive quality of enzymatically modified soy proteins. *International Journal of Food Sciences and Nutrition*, 56, 203–211. <https://doi.org/10.1080/09637480500146762>
- Buku, A., Condie, B. A., Price, J. A., & Mezei, M. (2005). [Ala¹²] MCD peptide: A lead peptide to inhibitors of immunoglobulin E binding to mast cell receptors. *Chemical Biology & Drug Design*, 66(3), 132–137. <https://doi.org/10.1111/j.1399-3011.2005.00281.x>
- Buku, A., Keselman, I., Lupyan, D., Mezei, M., & Price, J. A. (2008). Effective mast cell degranulating peptide inhibitors of the IgE/FcεRI receptor interaction. *Chemical Biology & Drug Design*, 72(2), 133–139. <https://doi.org/10.1111/j.1747-0285.2008.00684.x>
- Buku, A., Mendlowitz, M., Condie, B. A., & Price, J. A. (2003). Histamine-releasing activity and binding to the FcεRIα human mast cell receptor subunit of mast cell degranulating peptide analogues with alanine substitutions. *Journal of Medicinal Chemistry*, 46(14), 3008–3012. <https://doi.org/10.1021/jm0204892>
- Buku, A., Price, J. A., Mendlowitz, M., & Masur, S. (2001). Mast cell degranulating peptide binds to RBL-2H3 mast cell receptors and inhibits IgE binding. *Peptides*, 22(12), 1993–1998. [https://doi.org/10.1016/S0196-9781\(01\)00542-3](https://doi.org/10.1016/S0196-9781(01)00542-3)
- Cabanillas, B., Pedrosa, M. M., Rodríguez, J., González, Á., Muzquiz, M., Cuadrado, C., ... Burbano, C. (2010). Effects of enzymatic hydrolysis on lentil allergenicity. *Molecular Nutrition & Food Research*, 54, 1266–1272. <https://doi.org/10.1002/mnfr.200900249>
- Cabanillas, B., Pedrosa, M. M., Rodríguez, J., Muzquiz, M., Maleki, S. J., Cuadrado, C., ... Crespo, J. F. (2012). Influence of enzymatic hydrolysis on the allergenicity of roasted peanut protein extract. *International Archives of Allergy and Immunology*, 157, 41–50. <https://doi.org/10.1159/000324681>
- Clemente, A., Vioque, J., Sanchez-Vioque, R., Pedroche, J., & Millán, F. (1999). Production of extensive chickpea protein hydrolysates with reduced antigenic activity. *Journal of Agricultural and Food Chemistry*, 47, 3776–3781. <https://doi.org/10.1021/acs.jafc.5b05873>
- Codina, R., Lockey, R. F., Fernández-Caldas, E., & Rama, R. (1999). Identification of the soybean hull allergens responsible for the Barcelona asthma outbreaks. *International Archives of Allergy and Immunology*, 119, 69–71. <https://doi.org/10.1159/000024178>
- Eiwegger, T., Rigby, N., Mondoulet, L., Bernard, H., Krauth, M. T., Boehm, A., ... Mills, E. (2006). Gastro-duodenal digestion products of the major peanut allergen Ara h 1 retain an allergenic potential. *Clinical & Experimental Allergy*, 36, 1281–1288. <https://doi.org/10.1111/j.1365-2222.2006.02565.x>
- Gibbs, B., Amon, U., & Pearce, F. (1997). Spontaneous histamine release from mast cells and basophils is controlled by the cellular environment. *Inflammation Research*, 46(13), 25–26. <https://doi.org/10.1007/s000110050034>
- Helm, B., Spivey, A., & Padlan, E. (1997). Peptide blocking of IgE/receptor interaction: Possibilities and pitfalls. *Allergy*, 52(12), 1155–1169. <https://doi.org/10.1111/j.1398-9995.1997.tb02518.x>

- Horwitz, W. (2000). Official methods of analysis of AOAC International (17th ed.). Gaithersburg, Md.: AOAC International.
- Itoh, T., Garcia, R. N., Adachi, M., Maruyama, Y., Tecson-Mendoza, E. M., Mikami, B., & Utsumi, S. (2006). Structure of 8S α globulin, the major seed storage protein of mung bean. *Acta Crystallographica Section D: Biological Crystallography*, 62, 824–832. <https://doi.org/10.1107/S090744490601804X>
- Janeway, C. A., Travers, P. J., Walport, M., & Shlomchik, M. J. (2001). *The immune system in health and disease*. Immunobiology (5th ed., p. 563–597). New York, NY: Garland Science.
- Kasera, R., Singh, A., Lavasa, S., Prasad, K. N., & Arora, N. (2015). Enzymatic hydrolysis: A method in alleviating legume allergenicity. *Food and Chemical Toxicology*, 76, 54–60. <https://doi.org/10.1016/j.fct.2014.11.023>
- Kim, K., Kim, Y., Kim, H. Y., Ro, J. Y., & Jeoung, D. (2008). Inhibitory mechanism of anti-allergic peptides in RBL2H3 cells. *European Journal of Pharmacology*, 581(1–2), 191–203. <https://doi.org/10.1016/j.ejphar.2007.11.033>
- Knapp, S., Voadlo, D., Gao, Z., Kirk, B., Lou, J., & Withers, S. G. (1996). NAG-thiazoline, an N-acetyl- β -hexosaminidase inhibitor that implicates acetamido participation. *Journal of the American Chemical Society*, 118(28), 6804–6805. <http://doi.org/10.1021/ja960826u>
- Kopper, R. A., Odum, N. J., Sen, M., Helm, R. M., Stanley, J. S., & Burks, A. W. (2004). Peanut protein allergens: Gastric digestion is carried out exclusively by pepsin. *Journal of Allergy and Clinical Immunology*, 114, 614–618. <https://doi.org/10.1016/j.jaci.2004.05.012>
- Lack, G. (2012). Update on risk factors for food allergy. *Journal of Allergy and Clinical Immunology*, 129, 1187–1197. <https://doi.org/10.1016/j.jaci.2012.02.036>
- Lee, H. W., Keum, E. H., Lee, S. J., Sung, D. E., Chung, D. H., Lee, S. I., & Oh, S. (2007). Allergenicity of proteolytic hydrolysates of the soybean 11S globulin. *Journal of Food Science*, 72, C168–C172. <https://doi.org/10.1111/j.1750-3841.2007.00307.x>
- Li, W., Shu, C., Yan, S., & Shen, Q. (2010). Characteristics of sixteen mung bean cultivars and their protein isolates. *International Journal of Food Science & Technology*, 45, 1205–1211. <https://doi.org/10.1111/j.1365-2621.2010.02259.x>
- Li, G. H., Wan, J. Z., Le, G. W., & Shi, Y. H. (2006). Novel angiotensin I-converting enzyme inhibitory peptides isolated from Alcalase hydrolysate of mung bean protein. *Journal of Peptide Science*, 12, 509–514. <https://doi.org/10.1002/psc.758>
- Lindgren, M., Hällbrink, M., Prochiantz, A., & Langel, Ü. (2000). Cell-penetrating peptides. *Trends in Pharmacological Sciences*, 21(3), 99–103. [https://doi.org/10.1016/S0165-6147\(00\)01447-4](https://doi.org/10.1016/S0165-6147(00)01447-4)
- Malomo, S. A., & Aluko, R. E. (2016). In vitro acetylcholinesterase-inhibitory properties of enzymatic hemp seed protein hydrolysates. *Journal of the American Oil Chemists Society*, 93, 411–420. <https://doi.org/10.1007/s11746-015-2779-0>
- Mendoza, E. M. T., Adachi, M., Bernardo, A. E. N., & Utsumi, S. (2001). Mungbean [*Vigna radiata* (L.) Wilczek] globulins: Purification and characterization. *Journal of Agricultural and Food Chemistry*, 49, 1552–1558. <http://doi.org/10.1021/jf001041h>
- Misra, A., Kumar, R., Mishra, V., Chaudhari, B., Raisuddin, S., Das, M., & Dwivedi, P. (2011). Potential allergens of green gram identified as members of cupin superfamily and seed albumin. *Clinical & Experimental Allergy*, 41, 1157–1168. <http://doi.org/10.1111/j.1365-2222.2011.03780.x>
- Mittag, D., Vieths, S., Vogel, L., Wagner-Loew, D., Starke, A., Hunziker, P., ... Ballmer-Weber, B. (2005). Birch pollen-related food allergy to legumes: Identification and characterization of the Bet v 1 homologue in mungbean (*Vigna radiata*), Vig r 1. *Clinical & Experimental Allergy*, 35, 1049–1055. <https://doi.org/10.1111/j.1365-2222.2005.02309.x>
- Nielsen, P., Petersen, D., & Dambmann, C. (2001). Improved method for determining food protein degree of hydrolysis. *Journal of Food Science*, 66, 642–646. <https://doi.org/10.1111/j.1365-2621.2001.tb04614.x>
- Nowak-Węgrzyn, A., & Fiocchi, A. (2009). Rare, medium, or well done. The effect of heating and food matrix on food protein allergenicity. *Current Opinion in Allergy and Clinical Immunology*, 9, 234–237. <https://doi.org/10.1097/ACI.0b013e32832b88e7>
- Panda, R., Tetteh, A. O., Pramod, S. N., & Goodman, R. E. (2015). Enzymatic hydrolysis does not reduce the biological reactivity of soybean proteins for all allergic subjects. *Journal of Agricultural and Food Chemistry*, 63, 9629–9639. <https://doi.org/10.1021/acs.jafc.5b02927>
- Passante, E., & Frankish, N. (2009). The RBL-2H3 cell line: Its provenance and suitability as a model for the mast cell. *Inflammation Research*, 58(11), 737–745. <https://doi.org/10.1007/s00011-009-0074-y>
- Penas, E., Préstamo, G., Polo, F., & Gomez, R. (2006). Enzymatic proteolysis, under high pressure of soybean whey: Analysis of peptides and the allergen Gly m 1 in the hydrolysates. *Food Chemistry*, 99, 569–573. <https://doi.org/10.1016/j.foodchem.2005.08.028>
- Pessato, T. B., de Carvalho, N. C., Tavano, O. L., Fernandes, L. G. R., Zollner, R., & d. L., & Netto, F. M., (2016). Whey protein isolate hydrolysates obtained with free and immobilized Alcalase: Characterization and detection of residual allergens. *Food Research International*, 83, 112–120. <https://doi.org/10.1016/j.foodres.2016.02.015>
- Prioult, G., Pecquet, S., & Fliss, I. (2005). Allergenicity of acidic peptides from bovine β -lactoglobulin is reduced by hydrolysis with *Bifidobacterium lactis* NCC362 enzymes. *International Dairy Journal*, 15(5), 439–448. <https://doi.org/10.1016/j.idairyj.2004.09.001>
- Riascos, J. J., Weissinger, S. M., Weissinger, A. K., Kulis, M., Burks, A. W., & Pons, L. (2016). The seed biotinylated protein of soybean (Glycine max): A boiling-resistant new allergen (Gly m 7) with the capacity to induce IgE-mediated allergic responses. *Journal of Agricultural and Food Chemistry*, 64, 3890–3900. <https://doi.org/10.1021/acs.jafc.5b05873>
- Sampson, H. A. (2004). Update on food allergy. *Journal of Allergy and Clinical Immunology*, 113, 805–819. <https://doi.org/10.1016/j.jaci.2004.03.014>
- Shaheen, S., Harun, N., Khan, F., Hussain, R. A., Ramzan, S., Rani, S., ... Zafar, M. (2012). Comparative nutritional analysis between *Vigna radiata* and *Vigna mungo* of Pakistan. *African Journal of Biotechnology*, 11, 6694–6702. <http://dx.doi.org/10.5897/AJB11.3496>
- Sormus de Castro Pinto, S. E., Neves, V. A., & Machado de Medeiros, B. M. (2009). Enzymatic hydrolysis of sweet lupin, chickpea, and lentil 11S globulins decreases their antigenic activity. *Journal of Agricultural and Food Chemistry*, 57, 1070–1075. <https://doi.org/10.1021/jf803108c>
- Sung, D., Ahn, K. M., Lim, S. Y., & Oh, S. (2014). Allergenicity of an enzymatic hydrolysate of soybean 2S protein. *Journal of the Science of Food and Agriculture*, 94, 2482–2487. <https://doi.org/10.1002/jsfa.6583>
- Sung, D.-E., Lee, J., Han, Y., Shon, D.-H., Ahn, K., Oh, S., & Do, J.-R. (2014). Effects of enzymatic hydrolysis of buckwheat protein on antigenicity and allergenicity. *Nutrition Research and Practice*, 8, 278–283. <https://doi.org/10.4162/nrp.2014.8.3.278>
- Tewtrakul, S., & Itharat, A. (2007). Nitric oxide inhibitory substances from the rhizomes of *Dioscorea membranacea*. *Journal of Ethnopharmacology*, 109, 412–416. <https://doi.org/10.1016/j.jep.2006.08.009>
- Tewtrakul, S., Itharat, A., Thammaratwasik, P., & Ooraikul, B. (2008). Anti-allergic and anti-microbial activities of some Thai crops. *Songklanakarin Journal of Science & Technology*, 30, 467–473.
- Tsumura, K., Kugimiya, W., Bando, N., Hiemori, M., & Ogawa, T. (1999). Preparation of hypoallergenic soybean protein with processing functionality by selective enzymatic hydrolysis. *Food Science and Technology Research*, 5(2), 171–175. <https://doi.org/10.3136/fstr.5.171>
- van Boxtel, E. L., van den Broek, L. A., Koppelman, S. J., & Gruppen, H. (2008). Legumin allergens from peanuts and soybeans: Effects

- of denaturation and aggregation on allergenicity. *Molecular Nutrition & Food Research*, 52, 674–682. <https://doi.org/10.1002/mnfr.200700299>
- Verma, A. K., Kumar, S., Das, M., & Dwivedi, P. D. (2013). A comprehensive review of legume allergy. *Clinical Reviews in Allergy & Immunology*, 45, 30–46. <https://doi.org/10.1007/s12016-012-8310-6>
- Wang, Z., Li, L., Yuan, D., Zhao, X., Cui, S., Hu, J., & Wang, J. (2014). Reduction of the allergenic protein in soybean meal by enzymatic hydrolysis. *Food and Agricultural Immunology*, 25, 301–310. <https://doi.org/10.1080/09540105.2013.782268>
- Whitaker, J. R., Voragen, A. G., & Wong, D. W. (2003). *Proteolytic enzyme. Handbook of food enzymology* (1st ed., p. 969–994). New York, NY: Marcel Dekker.
- Whitcomb, D. C., & Lowe, M. E. (2007). Human pancreatic digestive enzymes. *Digestive Diseases and Sciences*, 52, 1–17. <https://doi.org/10.1007/s10620-006-9589-z>
- Yu, J., Ahmedna, M., Goktepe, I., Cheng, H., & Maleki, S. (2011). Enzymatic treatment of peanut kernels to reduce allergen levels. *Food Chemistry*, 127, 1014–1022. <https://doi.org/10.1016/j.foodchem.2011.01.074>
- Zhao, Y., Qin, G. X., Sun, Z. W., Zhang, B., & Wang, T. (2010). Stability and immunoreactivity of glycinin and β -conglycinin to hydrolysis *in vitro*. *Food and Agricultural Immunology*, 21, 253–263. <https://doi.org/10.1080/09540101003758954>

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