



## Hypoallergenic and immunomodulatory prospects of pepsin-educed soy protein hydrolysates

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### ABSTRACT

Consumption of soy protein and its products has increased significantly due to the increasing costs of animal proteins, esoteric supplement requirements, and several functional health benefits. Recently, the need to upgrade the functional features of soy protein led to new technologies including chemical and enzymatic processes. Hypoallergenic formulas and other immunomodulatory products are crucial targets of enzyme technology. Several proteases are used to achieve varying physiological properties and degrees of hydrolysis of soy protein, but reports on the use of pepsin are few, whereas it is among the paramount digestive proteases in the gastrointestinal tract of man. Bitterness and digestibility of soy protein hydrolysates have always been of concern, and there are ways of evading them. This review examines the potentials of pepsin-educed soy protein hydrolysates in relation to immunomodulatory and functional food products.

## Introduction

Soy protein is a plant-based protein known for its numerous health benefits including, among others, weight loss, sport nutrition supplement and cheap option for vegans. However, the consumption of soy-containing food products can cause severe and even fatal allergic reactions such as anaphylactic shock (Meinlschmidt et al., 2016), and other immune-related diseases including asthma and hay fever. The need to upgrade the functional features of soy protein recently led to more modern technologies, including modification of soybean genes, treatments with or without heat, as well as enzymatic hydrolysis (Meinlschmidt et al., 2016). Increasing market demands for organic foods or foods produced with the use of less chemical methods have brought enzymatic hydrolysis into the limelight. In addition, enzymatic hydrolysis is

widely applied to reduce loss of biological activity (Hsu, 2010). Soy protein, when hydrolysed, presents various physiological functions, including anti-inflammatory, anti-adipogenic, anti-allergic, antihypertensive, antioxidant, immunomodulatory, hypocholesterolemic, and ACE inhibitory properties (Penta-Ramos and Xiong, 2002; Tsou et al., 2010; Dia et al., 2014; Ezequiel et al., 2016; Ashaolu and Yupanqui, 2017; Ashaolu et al., 2017).

Soy and other food proteins have significant effects on the immune system, which entails many biological processes and structures that make resistance to disease possible in an organism. Antibodies play an important role in fighting antigens, such as bacteria, viruses, and toxins. The five subclasses of antibodies include Immunoglobulin A (IgA) that is present in high concentrations and mostly found in the mucous membranes; Immunoglobulin G (IgG), which is the most abundant type of antibody in all body fluids, and it protects against bacterial and viral infections.

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The other three are Immunoglobulin M (IgM), which can be found mainly in the blood and lymph fluid; Immunoglobulin E (IgE), particularly related to allergic reactions; and Immunoglobulin D (IgD), that exists in trace amounts throughout the body. Serum IgA reacts with FC receptor also known as CD89, and is expressed on immune effector cells, to initiate inflammatory reactions. This causes degranulation of eosinophils and basophils, as well as the phagocytosis of neutrophils, monocytes, and macrophages (Snoeck et al., 2006). There is an effective enhancement of mucosal immunity in gastrointestinal tract when immunomodulatory peptides are produced from plant proteins such as soy (Korhonen et al., 2003). The peptides may also assist proper functioning of the immune cells, which can be detected in lymphocyte proliferation, natural killer (NK) cell activity, antibody synthesis, and cytokine regulation (Hartmann and Meisel, 2007). Moreover, immunomodulatory peptides derived from tryptic hydrolysates of rice and soybean proteins act to stimulate superoxide anions (reactive oxygen species - ROS) which trigger non-specific immune defense systems (Kitts and Weiler, 2003). These reports indicated that soy protein hydrolysates might play a role in stimulating immunomodulatory and hypoallergenic activities. Until now, there have been very few reports on the immunomodulatory activity of soy peptides in animal models (Yamauch and Suetsuna, 1993; Yoshikawa et al., 1993). Xiang et al. (2008) prepared soy protein hydrolysates with various proteases, other than pepsin, and investigated their immunomodulatory potentials in vitro. Thus, this paper discusses the potentials of soy protein hydrolysates prepared with pepsin among other proteases, with much emphasis on its immunomodulatory and hypoallergenic attributes. Some concerns of soy protein hydrolysates as well as proffered solutions were also identified.

#### *Food allergies and immunity*

Approximately 220-250 million people are affected by food allergies (Pawankar et al., 2011). One in every three persons is allergic to certain foods, in developed societies (AAAAI, 2017). A food allergy is an immunological response to food, either IgE-mediated or not. Linear proteins are exposed to Th2 cells by antigen-presenting cells (APC). The APCs activate B cells to release antigen-specific IgE, which then attack the surface of the invading foreign antigen. Thereafter, receptors of the produced IgEs become bound to mast cells and degranulate so that cytokines, heparin, histamines and other mediators,

including many proteases are released into the environment (Stone et al., 2010). These in turn, produce the symptoms of allergy. Entrapped  $\beta$ -hexosaminidase in granules is also released in this process, and is useful in ascertaining the degree of degranulation in the study of allergic substances *in vitro* (Guo et al., 2009).

Generally, food allergens are proteins mediated by IgE. Structurally, proteins are either linear or sequential. The protein portion recognized by IgE is known as the epitope. Chronic allergy may result from the binding of IgE to epitopes of sequential amino acids (Sicherer, 2002). However, functional capabilities of proteins largely depend on their tertiary or three dimensional (3D) structure, which is highly susceptible to degradation by certain food processing techniques including the use of heat or chemical methods. In order to avoid loss of biological activity in food proteins, degradation of certain important amino acids such as cysteine, tryptophan, and serine, should also be avoided (Hsu, 2010). In such instance, enzymatic hydrolysis of food proteins is recommended. Extensively hydrolysed formulas with less than 5 kDa peptides are regarded as *semi-elemental* while partially hydrolysed formulas between 8 and 20 kDa peptides are termed *hypoallergenic* (El-Agamy, 2007; du Toit et al., 2010). Hydrolysed protein products can stimulate cellular immunity in experimental animals as well as the release of serotonin by the mast cells of sensitized normal rats (Fristché and Bonzon, 1990). Regardless of their bitter taste, hydrolysed formulas are well-accepted (Maldonado et al., 1998).

#### *Soy Protein*

Soy protein is extracted from dehulled or defatted soybean. It is usually produced as soy flour, concentrates, or isolates. Soy proteins are expected to contain at least 60-70% of the total soybean protein (Shewry et al., 1995). Due to the acceptance of health claims by many countries, soy protein is used in a variety of meat, beverage, cereal and other food industries. Soy protein isolate (SPI) is vastly utilized in food ingredients for nutritive or functional purposes to suit the needs of vegans and vegetarians who abstain from dairy products. However, soy allergy is one of the eight most common food allergies worldwide, mostly affecting children (FARE, 2017). Lactose-intolerant children may consume soy-based or any other non-lactose based formulas. Nevertheless, 0.4% of children are allergic to the soy protein used in their nutrition formula (Sicherer and Sampson, 2006). Not less

than 16 IgE-binding soy proteins having molecular masses of 7.5 to 97 kDa are identified with clinical allergy, and storage proteins Gly m5 (b-conglycinin) and Gly m6 (glycinin) recognized as the major allergen-containing components (Holzhauser et al., 2009; Amnuaycheewa and de Mejia, 2010). These concerns call for protein structural modification techniques, which eliminate soy protein allergenicity (Walter et al., 2016).

### Enzymatic hydrolysis

Many proteases have been used in the hydrolysis of SPI. Chymotrypsin, pomiferin, cucurbita, pepsin, papain, novozym, flavourzyme, alcalase, neutrase, and corolase have been used synergistically to achieve certain degrees of hydrolysis (Hrčková et al., 2002; Jung et al., 2004; Peñas et al., 2006; Tsou et al., 2010). The use of alcalase is more prevalent in many reports because of its economic prospects and assessment of the digestibility of proteins (Xiang et al., 2008; Meinlschmidt et al., 2016). Pepsin is uncommonly used in the previous reports on soy protein allergenicity and immunomodulation, whereas it is among the paramount biological proteases in man, which makes it an essential enzyme for studying gastrointestinal tract digestion and clinical nutrition.

Functional features of proteins in foods such as gelation, solubility, foaming and emulsifying attributes can be modified when hydrolysed, except that control measures should be taken to avoid extensive proteolysis that can destroy nutritive or functional benefits. Production of bitter-flavoured peptides is one of such (Jung et al., 2005). Chemical or enzymatic processes can achieve cleavage of peptide bonds or protein hydrolysis. Enzymatic hydrolysis ensures milder reaction conditions for better nutritional and chemical values, whereas chemical hydrolysis that includes acid or alkaline can be difficult to control, yielding products of modified amino acids (Maldonado et al., 1998; Castro et al., 2011). Alkaline hydrolysis can chemically reduce cystine, arginine, threonine, serine, isoleucine, and/or lysine content, and form unusual amino acid residues such as lysinoalanine or lanthionine (Provansal et al., 1975). Nonetheless, some major concerns with enzymatic process include inability of a single enzyme to effect complete hydrolysis, as well as enzyme's sensitivity to changes in protein structure (McGeagh et al., 2011; Tavano, 2013). There are often smaller molecular mass and improved functional properties resulting from protein

hydrolysis when compared to the parent or crude protein (Adler-Nissen and Olsen, 1979). This makes the hydrolysates much better because their intestinal absorption is more effective due to the increase of solubility (Ziegler et al., 1998).

### Choice of Proteases

Proteases, also known as peptide hydrolases, peptidases or proteolytic enzymes, are related to several biological systems. They are found in primitive organisms, and their mechanisms evolve from evolutionary processes, catalyzing the cleavage of amide linkages in proteins and peptides from monomers of 10 kDa to multimeric complexes; and are used as biological catalysts (Ivey and Little, 2008; Wensing et al., 2010). Substrate specificity is quite critical to a protease function. Some proteases are specific, binding to an array of substrates despite the high degree of precision in the alignment of the catalytic residues involved on the peptide bond cleavage, as the case with trypsin (Maupin-Furlow et al., 2005). Other proteases can be narrow or more specific as the case with Thrombin (Diamond, 2007). Proteolysis can occur within a polypeptide chain i.e. *endoprotease activity* or start from amino or carboxyl ends i.e. *exopeptidase activity*. This makes the activity and specificity of proteases relative.

In terms of catalytic potentials, proteases are mainly grouped into serine, aspartic, cysteine, threonine, glutamic and metal proteases. Others are oblique but described in past reviews (Vandeputte-Rutten and Gros, 2002). Despite these groupings, specificities of proteases are quite ambiguous. As found with soy protein, various hydrolysates can result from a protein when hydrolyzed with different proteases. This may result in significant differences in the degree of hydrolysis, amino acids composition and functionalities of the hydrolysates (Kamnerdpetch et al., 2007). Table 1 shows an overview of preferential cleavage of some proteolytic enzymes. Pepsin and papain are the only enzymes that have preference for hydrophobic amino acids cleavage. These amino acids are usually buried in the hydrophobic core of the protein, making their dissolution in aqueous environment practically impossible. Between these two, pepsin is found within (also produced from) the mammal digestive system, making it to be preferred above other enzymes that are derived from bacteria, such as alcalase (Betts and Russell, 2003).

**Table 1.** Preferential cleavage of proteolytic enzymes. Adapted from BRENDA (Tavano, 2013)

Protease	Hydrolysis reaction
Aminopeptidase A (EC 3.4.11.1)	Release of N-terminal Leu, but also may be other amino acids, including Pro, but not Arg or Lys.
Aminopeptidase B (EC 3.4.11.6)	Release of N-terminal Arg and Lys from oligopeptides when P1' is not Pro.
Carboxypeptidase A (EC 3.4.17.1)	Release of a C-terminal amino acid, but little or no action with -Asp, -Glu, -Arg, -Lys or - Pro.
Carboxypeptidase B (EC 3.4.17.2)	Preferential release of a C-terminal Lys or Arg
Chymotrypsin (EC 3.4.21.1)	Preferential release of N-terminal Tyr, Trp, Phe, Leu at P <sub>1</sub> position.
Papain (EC 3.4.22.2)	Preference for an amino acid bearing a large hydrophobic side chain at the P2 position.
Pepsin (EC 3.4.23.15)	Preferential cleavage: hydrophobic, preferably aromatic residues.
Thermolysin (EC 3.4.24.27)	Preferential release of C-terminal Leu and Phe at P <sub>1</sub> position.
Trypsin (EC 3.4.21.4)	Preferential release of N-terminal Arg and Lys at P <sub>1</sub> position.

### Pepsin mechanism of action

Kinetics and specificity of pepsin when cleaving the Phe(NO<sub>2</sub>)-Phe bond of GlyGly-Gly-Phe(NO<sub>2</sub>)-Phe-OMe in H<sub>2</sub>O and D<sub>2</sub>O led to the study of its mechanism as it exhibits a deuterium isotope effect in the breaking of a peptide bond (Hollands and Fruton, 1969). The binding of the carboxylate group (ECOO-) to the carbonyl-carbon of the amide group creates a tetrahedron. This undergoes a reversible exchange reaction that yields RCOOH and an imino-enzyme (ECO-NHR). As the intermediate reacts with carboxylic acid or water, transamidation by imino-transfer or formation of NH<sub>2</sub>R' occurs whilst reproducing ECOO- (Delpierre and Fruton, 1965). The essence of using the substrate of study was its spectrophotometrically proven connection with the specificity of pepsin. Thus, this mechanism serves as the basic catalytic pathway for all peptides including those of plant origin such as soy peptides.

### Soy protein hydrolysates (SPH)

*Soy protein hydrolysates* are products of enzymatic hydrolysis of soy protein that exhibit various physiological properties, which include hypolipidemic and hypocholesterolemic properties (Yoshikawa and Takahashi, 1993). The hydrolysates also possess some anti-allergic, anti-thrombotic, antioxidant, immunomodulatory, as well as angiotensin-converting enzyme (ACE) inhibition properties (Chen et al., 2004; Moure et al., 2006; Xiang et al., 2008; Ashaolu and Yupanqui, 2017;

Ashaolu et al., 2017). In addition, reduction of blood pressure, improvement in both arterial compliance and endothelial function, insulin resistance, and weight loss in obesity have also been reported (Gibbs et al., 2004; Ringseis et al., 2005; Chiang et al., 2006). Moreover, SPHs have been applied as clinical products, such as infant formulas and hospital diets for patients, as a food substitute. Table 2 presents an overview of the application of soy protein hydrolysates.

### Concerns on SPHs

Irrespective of proteases used (in this case pepsin), soy proteins are known to develop bitter flavour after hydrolysis, which is not suitable for consumers' acceptance. Thus, there is a need to control the hydrolysis of soy proteins. Taste receptor cells (TRCs) found in taste buds enable mammals (humans and animals) to perceive five basic tastes (sweet, bitter, salty, umami and sour), possibly as an adaptive reaction to avoid potentially poisonous foods, and this can facilitate product rejection (Glendinning, 1994). Amino acid profile of short peptides is responsible for their taste because peptides with low molecular weight and terminal hydrophobic amino acids exhibit bitter taste unlike their intact crude proteins (Sun, 2011). Peptides having higher amounts of isoleucine, tryptophan, tyrosine, phenylalanine and leucine are likely to be bitter, except that their quantification mechanism is sometimes inconsistent (Toelstede and Hofmann, 2008).

**Table 2.** Summary of application of soy protein hydrolysates (Sun, 2011)

Sources of hydrolysate	Enzymes	Conditions	Degree of hydrolysis	Results	References
SPI	Flavourzyme	2.5% (w/v) SPI dispersion, 1% (w/w of SPI) Flavourzyme, at 50 °C, pH 7.0 for 0.5–6 h	8%	Hydrolysates have a better suppression of adipogenesis in 3T3-L1 cell differentiation than intact SPI	Tsou et al. (2010)
SPI	A neutral protease from <i>Aspergillus oryzae</i>	Hydrolysis at pH 7, 50 °C for 1 h, with enzyme/substrate (E/S) ratios: 0.5/100 and 2/100	2% and 5.4%	The stability of foams and emulsions could be controlled and improved by addition of polysaccharides to soy protein hydrolysates	Martínez et al. (2007)
Traditional concentrate	Trypsin, pepsin	0.07% (w/v) soy protein concentrate dispersion, 0.0014% (w/v) trypsin/pepsin at 37 °C, pH 8 for 30, 60 and 90 min	–	All hydrolysates showed better extractability than the control, and trypsin significantly improved emulsifying properties	Barac et al. (2006)
Soy flour, SPC and SPI	A neutral protease derived from <i>Bacillus amyloliquefaciens</i>	6% and 10% (w/v) protein dispersions were hydrolysed using pH-stat method to determine DH at 50 °C and pH 7.0	2% and 4%	Protein solubility increased as DH increased. Emulsion stability improved for all 4% DH hydrolysates. Hydrolysed SPC had lower foaming capacity and stability	Jung et al. (2005)
SPI	Pepsin and papain	5% (w/v) SPI dispersions were hydrolysed by 0.001% (w/v) pepsin and 0.0025% (w/v) papain at pH 7.0 and 2.0, 70 and 37 °C for 30 min, respectively	–	The reduced- $\beta$ -conglycinin hydrolysate (RCH) retained more gel-forming ability than the reduced-glycinin hydrolysate (RGH). RGH with enhanced whippability could be used in foaming applications such as an egg white substitute	Tsumura et al. (2005)
Defatted Soy flour	Flavourzyme, Novozym, Alcalase	5% (w/v) soy flour dispersion at 40 °C, pH 7 for 8 h	Maximum 39.5%	Flavourzyme improves foaming and gelation property. Novozym decreases foaming stability	Hrčková et al. (2002)

To remove the bitterness in soy protein hydrolysates, activated carbon, silica gel, alcohol or chromatography can be utilized to selectively absorb bitter peptides from hydrolysates, but nutritional quality may not be guaranteed due to the loss of some amino acids (Saha and Hayashi, 2001). Otherwise, polyphosphates and specific amino acids such as Asp and Glu, are employed to mask the bitter taste of hydrolysates. Moreover, taste signaling can be modified by blocking bitter taste perception or else treat hydrolysates with peptidases (FitzGerald and O’Cuinn, 2006; Liman 2010). In addition, solubility of final products might be affected if transpeptidation or transglutaminase cross-linking are used in the production of debittered hydrolysates. Above all of these concerns and proffered remedies, bio-based methods seem more applicable for food products. Extensive hydrolysis of bitter peptides with proteases such as carboxypeptidase and aminopeptidase, use of *Lactobacillus* as a debittering adjunct starter, condensation reactions of bitter peptides are recommended, in addition to ultrafiltration (Saha and Hayashi, 2001).

#### Prospects of pepsin-educed SPH

Apart from infant formula production, soy proteins are used in meat, dairy and bakery industries.

Therefore, when hydrolysed into smaller peptides with better functionalities, they will be more effective as functional or immunomodulatory food products. Di- and tri-peptides obtained from enzymatically educed soy protein hydrolysates have been reported to be more effectively digested than either intact protein or free amino acids (Siemensma et al., 1993). Therefore, short chain soy peptides are of immense benefits in clinical nutrition. Also, the understanding of the fact that isoflavone aglycone forms (daidzein, genistein and glycitein), found in soy, are more readily absorbed than glucoside forms in humans suggests that transformation of bioinactive soy isoflavone glucosides to bioactive aglycones with proteases such as pepsin will improve nutritive and biological properties, for instance, in the prevention of chronic diseases (Izumi et al., 2000). Park et al. (2002) have applied  $\beta$ -glucosidases in the conversion of isoflavone glucosides to yield bioactive isoflavone aglycones in soy. Furthermore, some fractions of soy protein hydrolysates obtained with chymotrypsin serve as good source of protein for baby food as well as hypoallergenic formula (Barca et al., 2000).

Different proteases can bring about different degrees of hydrolysis to change allergenicity in proteins. For instance, there was a 65% reduction in IgE reactivity when roasted peanut protein was hydrolysed with flavourzyme for 5h, as compared with 100% decrease in IgE reactivity when treated with alcalase for just 30

mins (Cabanillas et al., 2012). Moreover, immunogenic capacities of chickpea and lentil proteins were lost after hydrolysis with pepsin and trypsin, while the antigenic property of sweet lupin protein hydrolysates reduced swiftly. Hydrolysates treated with pepsin yielded up to 23% of the antigenicity, but they were destroyed by trypsin hydrolysis in the same 30 mins interval (Sormus et al., 2009). Sometimes specificity of protease used, and the type of protein in question can produce entirely different results. Whey proteins treated with pepsin yielded hypoallergenic, vasodilatory and emulsifying hydrolysates (Mellinger-Silva et al., 2015; Lozano-Ojalvo et al., 2017). Kiwi allergens were highly susceptible to duodenal degradation after having been pepsinolysed into 4 and 6 kDa (Bublin et al., 2008). Hence, there might be a hope for kiwifruit allergic patients, as fresh insights into sensitization of their gut immune system are being provided. Uncooked sorghum and flour samples were more easily digested with pepsin *in vitro* when compared with cooked sorghum counterparts (Nunes et al., 2004).

Presently, investigations on pepsin-educed soy protein hydrolysates are exhaustive. Soy protein hydrolysed with pepsin was reported to exert some anti-allergic effects in RBL-2H3 cells (Ashaolu and Yupanqui, 2017). The SPH was also found to improve the immune system in rats and mice *ex vivo*, as well as modulate human colonic microbiota (Ashaolu et al., 2017, 2018). In a bid to modify inherent functional characteristics of soy protein, there were attempts to modify the isolate by selectively hydrolysing only a specific component such as glycinin or  $\beta$ -conglycinin. Tsumura et al. (2004, 2005) employed pepsin and papain to develop such processes and hydrolysates capable of meat tenderisation due to low viscosity and retained gel-forming ability when mixed with meat protein. In addition, soy isolate decomposed with trypsin and pepsin had improved emulsifying properties (Barac et al., 2006). Dia et al. (2014) observed that peptides in pepsin-pancreatin hydrolysates from soy products confer inhibitory effect on lipopolysaccharide-induced inflammation in macrophages, inferring that soy products can potentially be used to sustain health under inflammatory stress conditions. While investigating the hypocholesterolemic prospects of low-molecular weight soy protein hydrolysates, prepared with alcalase, Zhong et al. (2007) observed that the inhibition of cholesterol micellar solubility of soy peptides reduced by 4% after *in vitro* incubation with pepsin, which was not recorded in pancreatin digestion. Their results connote the use of these peptides as hypocholesterolemic ingredients meant for cardiovascular health risk populations. Finally, *in vitro*

digestion of soy and cowpea with pepsin produced fermented products that may play a role as sources of available nutrients for individuals suffering from digestive disorders, due to their digestibility, absorbability and solubility (Kiers et al., 2000).

The argument is that certain proteases such as alcalase or flavourzyme, are cost effective with increased protein hydrolysis in the shortest time (Xiang et al., 2008; Meinlschmidt et al., 2016). However, Ashaolu et al. (2017) reported the use of pepsin to be much more cost effective and bioactive than commonly used proteases. Further research is recommended to elucidate on their report.

## Conclusions

The fact that pepsin is a major protease in human gastrointestinal tract makes its applications in foods such as soy proteins/products, and clinical nutrition important. Soy protein is increasingly consumed; hence, there is a necessity to improve its nutritive and functional values, such as its flavour. Continuous improvement is quite important. Several proteases act in different ways due to their specificity and the protein in question, and pepsin is not out of the equation. Thus, soy protein prepared with pepsin has more functions and nutritional food applications including reduction of allergy, taste transformation and immunomodulatory prospects. More benefits abound in this area of study are still to be discovered.

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