

Review

Enzymatic hydrolysis of soy proteins and the hydrolysates utilisation

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Summary Soy proteins are very important protein source for human being and livestock. Enzymatic hydrolysis of soy protein can enhance or reduce its functional properties and improve its nutritious value. Soy protein hydrolysates were primarily used as functional food ingredients, flavour and nutritious enhancers, protein substitute, and clinical products. Conditions for hydrolysis were usually mild, whereas recently high pressure treatment attracted more interest. Degree of hydrolysis (DH) was usually between 1% and 39.5%. The main problem associated with proteolytic hydrolysis of soy protein was production of bitter taste, hydrolysates coagulation and high cost of enzymes. Bitterness reduction can be achieved by control of DH, selective separation of bitter peptides from hydrolysates, treatment of hydrolysates with exo-peptidases, addition of various components [adenosine monophosphate (AMP), some amino acids, monosodium glutamate (MSG), etc.] to block or mask the bitter taste, and modification of taste signalling. Hydrolysates coagulation can be resolved by selecting appropriate enzymes and by applying immobilisation technology the production cost can be reduced. Enzymatic hydrolysis also enhances bioactivity of soy proteins through conversion of glycosides to aglycones, increasing antioxidant and immunoregulatory properties. Finally, future works have been discussed.

Keywords Aglycones, debittering, degree of hydrolysis, enzymatic hydrolysis, glycosides, soy proteins.

Introduction

Soybean is a very important plant in the world. Beyond extraction of oil, it contains abundant high quality protein. The utilisation of soy protein is extensively studied for a long time. Soybean was originated in China and the custom of consumption soy foods, for instance tofu, soymilk, soy sauce, etc, has already taken place over thousands of years. Soy foods are also very popular in other Asian countries such as Japan, Korea, and Indonesia and the people greatly benefit from their nutritious and healthy effects.

Since 1980s, studies on soy foods, especially fermented soy foods have been attracting more and more attention and the mechanism of Chinese traditional fermented soy foods has been revealed. The very core of fermentation of soy foods is enzymatic hydrolysis of soy protein. Certain fermented soy foods for example fermented soy curd, soy pastes, and soy sauce, are actually enzymatic hydrolysis products. Through microbial fermentation, namely by hydrolysis of proteolytic enzyme produced by mould fungus and bacteria, the soy foods not only have improved flavour, but also enhanced absorptivity.

Therefore, fermented traditional Chinese soy foods are extremely nutritious and healthy.

Numerous new hydrolysed soy foods have been studied and developed based on this theory. In the research of Clemente *et al.* (1999), they indicated that among plant proteins, soybean is the source most widely used to obtain protein hydrolysates. In addition, the intake of soy products has increased in western countries due to their nutritional and healthy benefits (Anderson *et al.*, 1999).

There are two methods to hydrolyse proteins, acid hydrolysis and enzymatic hydrolysis. Appearance, solubility, flavour and biochemical safety of the hydrolysates vary with hydrolysis methods and DH. Currently, formation of chlorohydrins like 3-chloro-1, 2-propanediol (MCPD) and 1, 3-dichloro-2-propanol (DCP) in vegetable protein (HVP) hydrolysis due to strong acid treatment, especially concentrated hydrochloric acid hydrolysis, has been a serious concern in many countries to use the HVP clinically (Collier *et al.*, 1991).

Now researchers and manufacturers tend to apply safe and mild methods such as enzymatic hydrolysis to replace strong acid hydrolysis. Compared to acid hydrolysis, enzymatic hydrolysis of vegetable proteins has few or no undesirable side reactions or products

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produced, is safer, and provides a more uniform product through more specific reactions (Campbell *et al.*, 1996). In addition, the functionality of the final product can be controlled by selection of specific enzymes and reaction conditions (Madsen *et al.*, 1997; Darwicz *et al.*, 2000). So it is a very promising method to produce functional and nutritious food ingredient, or develop clinical health foods. With development of biochemical technology, purified proteases have become available. This also promotes the development of new enzymatically hydrolysed soy protein products.

Soy foods can unfortunately induce allergies in some consumers despite all these advantages (Peñas, 2006). Allergies are abnormal inflammatory responses to the immune system and the intolerance of soy protein may cause different clinical syndromes, such as rhinitis, urticaria, asthma, atopic dermatitis, anaphylactic shock or even death. Soybean has substances that can produce allergic reactions but, in this aspect it is behind other foods such as peanuts, tree nuts, milk, eggs, shellfish, fish and wheat (Peñas, 2006). A report indicated that the combination of both enzymatic hydrolysis and high pressure (HP) treatment reduced its allergenicity (Peñas, 2006).

Utilisation of soy protein hydrolysates

Soy proteins are used in foods as functional and nutritional ingredients and as a substitute for animal-derived proteins from milk, meat and eggs, and enzymatic hydrolysis of food proteins is one of the most efficient methods for improving and expanding their functional properties (Cai *et al.*, 1996). Low denaturation soy protein isolate (SPI) has good gelation, emulsifying, foaming and water absorption properties (Utsumi *et al.*, 1997), enzymatic hydrolysis of SPI can further improve these functional characteristics.

First, soy protein hydrolysates can be used as functional food ingredients. Native soy protein isolates are mainly composed of 7S and 11S globulins and show high solubility (>90%) at alkaline conditions (Thanh & Shibasaki, 1976). At pH < 7, the solubility of native soy isolates decreases to a minimum close to the isoelectric point ($pI = 4.5$; Pearson, 1983). Protein fortification of acidic foods, having a pH in the range of 2–6, is also complicated by the unavailability of protein exhibiting satisfactory functional properties, particularly solubility. The low solubility of soy proteins in acidic media restricts their utilisation in foodstuffs of moderate acidity (citric beverages, dressings, etc.), especially when the required functional properties depend on solubility, e.g. foaming and emulsifying properties.

A useful alternative to increase the protein solubility of both native and modified soy protein isolates is through proteases induced hydrolysis (Achouri & Wang, 2001; Molina & Wagner, 2002). Many researchers have

reported an improvement in some functional properties of plant proteins using limited enzymatic hydrolysis, but the solubility was still poor at slightly acidic pH (Achouri *et al.*, 1999). Research shows that the soy proteins solubility can be increased using enzymatic hydrolysis followed by succinylation without apparent damage to digestibility, and the improvement in enhancing solubility of succinylated soy protein hydrolysates at acidic pH was attributed to changes in the physicochemical characteristics of the polypeptide mixture resulting from altered conformation and an increase in net negative charge (Achouri & Wang, 2001). Ortiz & Wagner (2002) performed a series of hydrolysis experiments at pH 4.5 and observed that the solubility increment were between 20% and 26%. They believed the hydrolysates could be used for preparation of foodstuffs of moderate acidity.

Limited enzymatic hydrolysis of soy proteins by proteases can also significantly improve foamability (Ortiz & Wagner, 2002; Tsumura *et al.*, 2005). This could be due to the exposure of hydrophobic areas and increased molecular flexibility of polypeptides that increases the affinity for the interface and the adsorption rate (Ipsen *et al.*, 2001; Conde & Patino, 2007). Nonetheless, researchers (Vioque *et al.*, 2000) observed that limited hydrolysis may improve foaming capacity but reduce foam stability. Also, at high level of DH, enzymatic hydrolysis was found to increase solubility while lower foaming ability of SPI. Due to the high DH, the decrease in molecular size can be expected to reduce the ability of the polypeptides to interact at the interface so that less viscoelastic films will cause a decrease in foaming capacities (Achouri *et al.*, 1999) and foam stability (Martínez *et al.*, 2007). These authors (Martínez *et al.*, 2011) further investigated polysaccharide addition for enhancing foaming properties of soy protein hydrolysates and concluded that the DH, surface-activity of polysaccharide, concentration of both macromolecules, contribution of polysaccharide consistency to bulk viscosity and interfacial interactions between biopolymers all contributed to foaming properties and the addition of polysaccharides took effect in a complicated way.

Soy proteins have also been subjected to enzymatic hydrolysis as an alternative of improving the emulsifying properties (Cai *et al.*, 1996; Jung *et al.*, 2005). A mayonnaise emulsion preparation that is stabilised by hydrolysed soy proteins has also been previously reported (Mikami *et al.*, 1981) and they have been further developed to a commercial whipping protein. These products could apply as components of diet foods and fortifiers enriching concentrated soups and drinks, as well as components of seasoning mixtures. Extrusion pre-treatment has also been proved to be an efficient method to improve emulsifying properties of enzymatic hydrolysed SPI (Surówka *et al.*, 2004a; b). Chen *et al.* (2011) investigated the combination of extrusion

pre-treatment and controlled enzymatic hydrolysis on the physicochemical and emulsifying properties of soy protein isolates and observed that extrusion of pre-treated SPI hydrolysates (ESPIH, 9.1% DH) was capable of producing a very fine emulsion ($d_{32} = 0.42 \mu\text{m}$, $d_{43} = 2.01 \mu\text{m}$) which remained stable over a long term quiescent storage. They suggested that the main reasons for the greatly improved emulsifying capability of ESPIH were attributed to significantly increased protein solubility and decreased molecular weight.

Surówka *et al.* (2004b) used Alcalase and Esperase to hydrolyse extruded soy protein concentrate (SPC) and obtained hydrolysate with significantly increased emulsifying activity index (EAI), foaming yield, and foam stability compared to non-hydrolysed extruded SPC. Like other plant protein products, soy protein hydrolysates may also be used in confectionery, and bread-making industries, as well as in the meat processing industry (Surówka *et al.*, 2004b).

It is worth noting that some protein hydrolysates, such as hydrolysed vegetable protein (HVP) are employed as flavour enhancers. Soy protein hydrolysates seem especially suitable for making flavour enhancer due to their abundance and competitive price and numerous foods have already been HVP added to improve their flavour. In fact, this is the most extensive application of soy protein hydrolysates. Due to the reduced antigenic activity of protein hydrolysates, their development for use in peptide-based hypoallergenic infant formulas has also been reported (Cordle, 1994).

Second, soy protein hydrolysates can be employed as clinical products. Protein hydrolysis is a common food process utilised for the production of hospital diets for patients and protein liquids/powders for weight control diets (Adler-Nissen, 1986). Frokjaer (1994) reported protein hydrolysates are used in clinical applications, such as geriatric products, high-energy supplements, weight-control and therapeutic or enteric diets. Extensive hydrolysis of protein is required for HVP production to obtain a high amino acid level. Carefully designed partial hydrolysis may be acceptable for hospital diets (Adler-Nissen, 1986).

Third, soy protein hydrolysates can be applied as some food substitute. Olsen (1984) and Boyce *et al.* (1986) invented egg white substitutes produced by enzyme modified soy protein. The DH was between 1% and 8%. The hydrolysed soy protein material exhibited both superior whipping and emulsifying abilities, had good nutritional value, and no bitter taste.

The development of enzymatic hydrolysed soy protein products approached a climax in China in the period of 1980–1990s. Some of the most famous brands such as Yang Zhenhua-851, San Zhu oral liquid were so successful that tens of millions of consumers have consumed them. The problem was, although they were healthy foods, manufacturers advertised their products

as medicine and emphasised the healing effect, such as to cure cancer, psoriasis, and to build up people's health. Gradually people found the efficacy was not as what have been advertised, thus finally these products did not attract public interest. Presently soy enzymatic hydrolysates are used as ingredients in nutritious beverage, infant food, and in athletes and patient food formulae. For example, Nestle uses soy enzymatic hydrolysates as an ingredient in its infant powder. Partially enzymatic hydrolysed soy proteins are extensively employed in milk powder to reduce cost and provide balanced nutrition. In feed industry, soy protein hydrolysates have been applied in fishmeal as major nutritious ingredients. A summary of the applications of soy protein hydrolysates is presented in Table 1.

Selection of proteases used

Most of the proteases used in protein hydrolysis can be applied to soy hydrolysis. At present, some of the enzymes employed in the production of hydrolysates for food use are from plant or microbial origin, as bromelain, papain or ficin (Lahl & Braun, 1994; Ortiz & Wagner, 2002) due to their large scale availability, while enzymes of animal origin, due to their limited supply, are usually too expensive for food processing thus generally used in laboratory experiments. Proteolytic enzymes that have been investigated include pepsin, papain, trypsin, chymotrypsin, cucurbita, hieronymin, pomiferin plus various bacterial and fungal proteases (Ortiz & An, 2000; Jung *et al.*, 2004).

Peñas *et al.* (2006) reported using four proteases Alcalase (E.C. 3.4.21.62), Neutrase (E.C. 3.4.24.28), Corolase 7089 (B.C. 3.4.24.28) and Corolase PN-L 100 (E.C. 3.4.21.63) to hydrolyse soy-whey during high pressure (HP) treatment and indicated that Alcalase possessed the highest DH whatever pressure used. Hrecková *et al.* (2002) applied Flavourzyme 1000 L, Novozym FM 2.0 L (subtilisin Carlsberg) and Alcalase 2.4 L FG to hydrolyse defatted soy flour and found that each enzyme degraded both β -conglycinin and glycinin. 1.398 neutral proteinase (from *B. subtilis* as 1.398) (Guo *et al.*, 1996), protease D3 (Kodera *et al.*, 2006), Alcalase, Flavourzyme, and peptidase NP-2 (Lee *et al.*, 2001) were employed to hydrolyse soy protein or defatted soy flour. In addition, lactic acid bacteria were also used to hydrolyse soy protein by the enzymes they produced (Aguirre *et al.*, 2008).

Transglutaminase (TGase) has also been used in improving functionality of soy protein hydrolysates. Walsh *et al.* (2003) observed that between pH 3.0 and 5.0, SPI hydrolysates, hydrolysates of cross-linked SPI and the cross-linked products of SPI hydrolysates displayed significant ($P < 0.05$) increases in solubility compared to unmodified SPI. Combined use of TGase cross-linking and hydrolysis has the potential for

Table 1 Summary of application of soy protein hydrolysates

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 <i>et al.</i> (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	Martinez <i>et al.</i> (2007)
Liquid effluent from soy concentrate	Flavourzyme	Enzyme/protein ratios were 10, 50, 100 and 500 LAPU/g protein and hydrolysed for 0.5, 1, 2 and 4 h in stirred vessels at 50 °C	19.4%–63.4%	The fraction (molecular weight <10 kDa) presented the highest antioxidant activity. Hydrolysates showed higher reducing power, higher antioxidant potency in emulsion than the original fractions	Moure <i>et al.</i> (2006)
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 <i>et al.</i> (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 solubilities of all substrates increased as DH increased. Emulsion stability was improved for all 4% DH hydrolysates. Hydrolysed SPC had lower foaming capacity and stability	Jung <i>et al.</i> (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- β -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 <i>et al.</i> (2005)
Soy flour extrudate	Neutral proteases of <i>Bacillus</i> origin, Protamex and Neutrase	A response surface design was used at pH from 4.4 to 8.0, temperature from 35 to 70 °C, and water added to the raw material in a ratio of 11.5, 13.3, 15.7, 19.0, 24.0 and 32.3 g H ₂ O/g protein	–	Limited hydrolysis of extruded soy flour enhanced solubility and foaming properties and features conducive to the formation of emulsions. The hydrolysates may be used as components of diet foods and fortifiers enriching concentrated soups and drinks in protein, as well as seasoning mixtures	Surówka <i>et al.</i> (2004)
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	Hrcková <i>et al.</i> (2002)

generating function improved soy protein ingredients for the application in low pH food and beverage.

Conditions for enzymatic hydrolysis

For the substrate for enzymatic hydrolysis, typically soy protein isolate (SPI), defatted soy flour (DSF), nonfat soy flour (NSF), soymilk, even waste of producing Tofu (soybean whey) are employed. Hydrolysis of soy proteins was usually conducted at mild temperature and atmosphere pressure. However, high pressure (HP) treatment was employed to increase efficiency of protein hydrolysis (Stapelfeldt *et al.*, 1996; Maynard *et al.*, 1998). An increase of DH by pepsin, trypsin and chymotrypsin was observed on soybean when the HP treatment was applied before or during hydrolysis. According to Kajiyama *et al.* (1995), the HP treatment dissociates the proteins and as a result, the DH increases. Different pressure treatments for soy whey proteins were studied to identify optimum processing conditions. On the basis of previous studies, enzymatic hydrolysis of soy whey proteins subjected to pressures of 100, 200, 300 and 400 MPa were investigated (Peñas & Préstamo, 2002). A research result showed that HP treatments increased the DH of trypsin, chymotrypsin and pepsin and 100 MPa was better than normal air pressure (Peñas *et al.*, 2004). A sharp decrease of DH occurred when the proteins were treated at 300 or 400 MPa and this was related to aggregation of proteins by high pressure (Peñas & Préstamo, 2002).

In the study of Peñas *et al.* (2006), four proteases Alcalase, Neutrase, Corolase 7089 and Corolase PN-L 100 were employed to hydrolyse 5% (w/v) soy-whey (atmospheric pressure, 100, 200, and 300 MPa, 15 min). Their results showed that HP treatments increased the DH by these proteases. 200 and 300 MPa proved to be optimum pressure to enhance the proteolysis. In addition, the allergen Gly m 1 content decreased by the combination of enzymatic hydrolysis and HP treatment thus allergenicity of soy protein hydrolysate could be reduced.

Mild acid pre-treatment was also reported to benefit enzymatic hydrolysis of soy protein. Lee *et al.* (2001) employed 0.2 N HCl to pre-treat defatted soy flour slurry, and then use Alcalase and flavorzyme to hydrolyse the soy proteins. Their results showed that with mild acid pre-treatment, the DH increased from 9.6% (control) to 14.3%.

Ultrasonic wave treatment was another method used to increase the DH of soy protein. Hu & Zheng (2005) employed 40 kHz, 128 W ultrasonic waves to treat slurry of defatted soy protein flour, and then employed alkaline protease 2709. The DH of different treatments increased 6.48–30.86% in comparison to control.

Extrusion pre-treatment has also been applied to increase the accessibility of enzymes to SPI so that

improved emulsifying properties can be achieved (Surówka *et al.*, 2004a; b). Chen *et al.* (2011) observed that extrusion pre-treatment of SPI before enzymatic hydrolysis was capable of producing a very fine emulsion ($d_{32} = 0.42 \mu\text{m}$, $d_{43} = 2.01 \mu\text{m}$) which remained stable over a long term quiescent storage.

The flavour of hydrolysates can also be controlled by adjusting hydrolysis condition. Aaslyng *et al.* (1998) compared the aroma characteristics of acid-hydrolysed and enzyme-hydrolysed soy proteins and reported that manipulation of the hydrolysis conditions was necessary to increase the levels of desirable aroma components, for example sulphur compounds and Strecker aldehydes, and to reduce possible off-odours, such as hexanal and 2-methoxyphenol.

In conclusion, the conditions for enzymatic hydrolysis of soy proteins are mild, at moderate temperature and atmosphere pressure, and the pH needs to be adjusted to the optimal pH for enzyme activity. According to some research results, high pressure, heat, acid, ultrasound and extrusion pre-treatments are beneficial to enzymatic hydrolysis due to the action of loosen the protein structure. Moreover, by controlling hydrolysis conditions, it is possible to control the flavour of SPI hydrolysates.

DH controlled

Since some amino acids taste bitter, with development of soy protein hydrolysis, bitter amino acids originally hidden in the internal of the protein structure expose to the surface of hydrolysed short peptide chains. When the intensity of bitterness exceeds the threshold value, a bitter taste appears.

Controlling the degree of enzymatic hydrolysis is essential for reducing the bitterness. pH-stat technique was applied to determine the reaction rate and maintain the pH at a constant level, but pH-stat only works at neutral to alkaline range or below pH 3 (Adler-Nissen, 1986).

Usually the DH is in the range of 1–25%. Guo *et al.* (1996) indicated that the maximum extent of hydrolysis of soy protein by immobilised protease is about 23%. Hrecková *et al.* (2002) achieved the highest DH (39.5%) using protease Flavourzyme.

Methods of analysis

Usually, in soy protein hydrolysis, degree of hydrolysis (DH), average peptide chain length and average molecular weight are the most often used parameters to describe extent of hydrolysis. To determine the DH, pH-stat method (Jacobsen *et al.*, 1957), trinitrobenzenesulfonic acid (TNBS) method (Alder-Nissen, 1979), ninhydrin method (Pearce *et al.*, 1988), osmometry method (Adler-Nissen, 1984), monitoring soluble nitro-

gen content (Church *et al.*, 1983; Nielsen *et al.*, 2001), *O*-phthaldialdehyde (OPA) method can be applied; Lee *et al.* (2001) estimated average peptide chain length from DH to determine soy protein hydrolysis; gel permeation chromatography (Ortiz & An, 2000; Morten *et al.*, 2002) and SDS-PAGE (Barac *et al.*, 2006; Aguirre *et al.*, 2008; Zhao & Hou, 2009) based on investigation of average molecular weight were also used to determine soy protein hydrolysis.

Although DH is extensively used as an index for protein hydrolysis, Jung *et al.* (2005) indicated that the peptide profiles of the endo-peptidase hydrolysates for the soy flours showed different extents of enzymatic attack than did the SPC and SPI at the same DH. They concluded that when comparing different hydrolysed soy protein products prepared from various forms, DH alone was not a good indicator for the extent of protein hydrolysis.

Problems associated with soy protein hydrolysis and the resolutions

In general, three major problems are associated with enzymatic hydrolysis of soy protein that have limited its utilisation.

First, the extent of the reaction must be carefully controlled. Soy proteins, in particular, often develop an intensely bitter flavour when hydrolysed (Adler-Nissen, 1984). Since foods flavour has to meet consumers' acceptance, the formation of bitter peptides during partial hydrolysis of soy proteins must be controlled.

Mammals can perceive five basic tastes (sweet, sour, bitter, salty, and umami) by taste receptor cells (TRCs), which are assembled into taste buds. Recent molecular studies have discovered that the five basic tastes are mediated by specific receptors expressed on TRCs. Receptor candidate related to bitter taste perception was the receptor T1R2 (formerly known as T2R), which was cloned and identified as member of class C G-protein-coupled receptor (GPCR) subfamily. Researchers (Ruiz-Avila *et al.*, 1995; Kusakabe *et al.*, 2000) indicated that TRCs perceive bitter stimuli by the T2R GPCRs, triggering a G-protein-mediated signalling cascade. In TRCs, several G-protein α -subunits have been identified, including G α i2, G α i3, G α 14, G α 15, G α q, G α s, α -transducin, and α -gustducin (Maehashia & Huang, 2009). Not all α -gustducin-expressing TRCs possess bitter receptors, although nearly all T2Rs are in α -gustducin-expressing TRCs. It is believed that a multigene family of G-protein-coupled receptors, T2R (TAS2R or TRB, $n = 26$) receptors and a G-protein α subunit (G α), gustducin, to be key molecules for bitter sense perception in mammals (Ueda *et al.*, 2003) and each of the T2R receptors likely recognises one compound or a few structurally related compounds (Chandrashekar *et al.*, 2000; Mueller *et al.*, 2005).

The flavour of the hydrolysed vegetable proteins is in general associated with the presence of free amino acids, smaller peptides, salt, and various volatile compounds (Aaslyng *et al.*, 1998). The taste of the smaller peptides is a function of their amino acid composition. A peptide containing hydrophobic amino acids generally has a bitter taste (Ney, 1979). The bitterness seems to be especially problematic if peptides with a low molecular weight are produced. During the tryptic hydrolysis of soybean 11S glycinin, Kim *et al.* (1999) observed that the formation of bitterness peptic fractions primarily of 0.36–2.10 kDa were responsible for the bitterness. However, to some extent this condition depends on the protein and on the specificity of the enzyme.

The mechanism of bitterness has been extensively studied. Enzymatic hydrolysis of proteins frequently results in bitter taste, which is due to the formation of low molecular weight peptides, whose terminal amino acids are hydrophobic, such as isoleucine, tyrosine, phenylalanine, and tryptophan, thus the bitterness is related to their hydrophobicity (Yeom *et al.*, 1994; Saha & Hayashi, 2001). In intact proteins, they do not display bitterness as hydrophobic amino acid residues are usually buried inside the molecule thus have no chance to contact with taste receptors when ingested. Hydrolysis disrupts protein structure hence enables hydrophobic amino acid residues to contact with taste receptors. Peptide bitterness was quantified by Ney (1971) on the basis of the hydrophobicity of the side-chains within a given peptide sequence and was calculated from the free energy of transfer of an amino acid side chain from ethanol to water. Ney reported that peptides with hydrophobicity (Q) values > 1400 cal per mole and molecular masses < 6 kDa display bitterness. Nevertheless, peptides with Q values < 1300 cal mol $^{-1}$ along with peptides with Q values > 1400 cal mol $^{-1}$ and molecular masses > 6 kDa would not be bitter. Therefore, peptides < 6 kDa having a high content of Leu, Pro, Phe, Tyr, Ile and Trp residues are likely to be bitter. However, inconsistency has been reported between some peptides' bitterness and their Q values (Toelstede & Hofmann, 2008). Hence to predict the bitterness of a peptide simply on the basis of its Q value sometimes is inadequate.

According to Okai's theory, two functional groups were necessary to produce bitterness: a pair of hydrophobic groups or a hydrophobic and a basic group (Tamura *et al.*, 1990). It has also been proposed that the sensation of bitterness was exerted by chemical compounds with a hydrophobic region and one hydrophilic group spaced 0.3 nm apart (Belitz & Jungel, 1979). However, bitterness often decreases at high degrees of hydrolysis; this implies that bitterness can be reduced by splitting bitter peptides into free amino acids (Xu, 1997). Further study with certain bitter peptides isolated from haemoglobin hydrolysates demonstrated that hydrolysis of these peptides to free amino acids considerably

decreased the hydrophobicity and reduced or eliminated their bitterness (Aubes-Dufau & Combes, 1997). However, this is not the case in soy protein. In the study of Cho *et al.* (2004), it was shown that the hydrophobicity data based on Q values do not support Ney's Q rule as a predictor of bitterness for soy peptides and hydrolysis of soy protein to free amino acids did not decrease its bitterness.

Methods for debittering of protein hydrolysates include: selective separation of bitter peptides from hydrolysates (using activated carbon or chromatography to absorb bitter peptides), addition of various components (adding polyphosphates, specific amino acids such as Asp and Glu, α -cyclodextrins) to mask the bitter taste of hydrolysates, treatment of hydrolysates with exo- and endo-peptidases (Pedersen, 1994; FitzGerald & O'Cuinn, 2006), and to modify taste signalling (to block bitter taste perception) (Liman, 2010). However, selective separation of bitter peptides results in the loss of some amino acid residues from hydrolysates thus decreases the nutritious value of the hydrolysates. Transpeptidation and transglutaminase cross-linking are also used in the debittering of hydrolysates, but these protocols may reduce solubility of final products hence not appropriate for certain products.

Selective separation includes treatment with activated carbon, extraction with alcohol, isoelectric precipitation, chromatography on silica gel, hydrophobic interaction chromatography (Saha & Hayashi, 2001). Most of these methods do not seem applicable for producing food products. Bio-based methods include further hydrolysis of bitter peptides with enzymes such as aminopeptidase, alkaline/neutral protease and carboxypeptidase, condensation reactions of bitter peptides using protease, and use of *Lactobacillus* as a debittering starter adjunct (Saha & Hayashi, 2001). Many of the unwanted effects including bitterness can also be overcome by using ultrafiltration (Cheryan *et al.*, 1984). These methods seem more practical because to separate and get rid of the bitter peptides and amino acids is not easy and the balance of amino acids will be disrupted that results in soy protein nutritional values decrease.

Adler-Nissen (1984) introduced the concept of degree of hydrolysis (DH) to control the bitterness of partially hydrolysed proteins. The mixture of endo-peptidase and exo-peptidases has been used for food protein hydrolysis in order to reduce the bitterness as well as to increase the DH (Izawa *et al.*, 1997).

Alcalase was employed to hydrolyse soy protein by LovsinKukman *et al.* (1996) to prepare soluble and isoelectric soluble hydrolysates using a DH of 3–15%. They concluded that the main reason for the bitterness treated by Alcalase was hydrophobic bitter peptides of relative molecular weight less than 1000 Da.

To reduce bitterness, Yeom *et al.* (1994) investigated chemical modification of lysine. Isolated soy protein

(ISP) was treated with N-acetylimidazole for acetylation of lysine and tyrosine. *O*-acetyl tyrosine was deacetylated at pH 11. Sensory analyses indicated that bitterness of lysine-acetylated ISP decreased in comparison with hydrolysates of control ISP.

Enzymatic debittering is another method to reduce bitterness. Nishiwaki *et al.* (2002) treated bitter peptide solutions of soy protein hydrolysates with an aminopeptidase from the edible basidiomycete *Grifola frondosa*. It was shown that as the incubation time elapsed, the amount of free amino acids released increased and the bitterness of the hydrolysates decreased. Hydrophobic amino acids such as valine, leucine, phenylalanine, tyrosine, and isoleucine were preferentially released from the bitter solutions by the action of the aminopeptidase.

Kodera *et al.* (2006) purified a novel soy protease D3 from germinated soy cotyledons to produce low bitterness soy protein hydrolysates. They used proteinase D3, subtilisin, pepsin, trypsin, and thermolysin to hydrolyse soy protein. They observed that the proteins hydrolysed by protease D3 were significantly less bitter than the other enzymatic hydrolysates.

Recent research has focused on three alternatives to reducing or eliminating bitterness: bitter blocking (transduction pathways), bitter masking (psychological interference with bitterness perception) (Ming *et al.*, 1999), and modification of taste signalling (to block bitter taste perception) (Liman, 2010). One method of blocking bitter taste involves the use of sodium salts. Additional blockers of bitterness including phosphatidic acid- β -lactoglobulin, glutamate, adenosine monophosphate (AMP), and monosodium glutamate (MSG) have also been recognised. It may be possible to predict the efficiency of these blockers with advance in understanding the molecular mechanisms underlying bitter taste perception and how bitter blockers function to suppress bitterness.

For bitter tastes, the perception process is initiated by apically located taste receptors that signal through a G protein-dependent phospholipase C (PLC) based second messenger signaling cascade (Margolskee, 2002; Montmayeur & Matsunami, 2002; Medler & Kinnamon, 2004). The ion channel TRPM5, which is specifically expressed in taste cells is likely to be the downstream target of this cascade (Perez *et al.*, 2002) and is essential for bitter taste responses (Zhang *et al.*, 2003; Damak *et al.*, 2006). It is primarily expressed in taste cells, provides an attractive target to modify bitter taste.

Second, the cost of enzymes in conventional, batch-type hydrolytic systems is high. The proteins and the enzymes are typically mixed in suspension at the optimum temperature and pH for several hours. When the desired DH is achieved, the enzymes are inactivated immediately by heating. Therefore, the enzymes can only be used once, and the heat treatment also increases the cost of production (Cheryan & Deeslie, 1984).

Enzyme immobilisation has been developed rapidly recent years. By attaching enzyme to a solid support such as silica, alumina, or iron oxide, it is immobilised. The protein flows through a column containing the immobilised enzyme and hydrolysed. The extent of hydrolysis is controlled essentially by the flow rate or length of time in the column. A major disadvantage is that immobilisation causes a large drop in enzyme activity (Cheryan & Deeslie, 1984). However, this technology provides an alternative to increase production efficiency and reduce cost.

In some cases soy protein coagulates during treatment with proteases (Aoyama *et al.*, 20005). It was reported that water-soluble soy protein isolate prepared from defatted soybean meal showed aggregation and gelation by stem bromelain hydrolysis (Fuke *et al.*, 1985). Bromelain attacks the basic subunit of 11S globulin and converts them to low molecular weight fragments and aggregation develops with an increase of the fragments. This coagulum is insoluble and decreases the yield of the soluble hydrolysates. The coagulation by protease is different from that mediated by Mg^{2+} and Ca^{2+} in preparing tofu, it may be expected that the coagulum has a possibility to be used as a foodstuff with a novel texture and functional property (Inouye *et al.*, 2002). It seems like to select protease which does not produce coagulates is the best method for preventing the coagulation.

Prospect of soy protein hydrolysates

Soy proteins have been found widespread used as a fat and protein replacer in dairy, meat and bakery products as well as in infant formula. Enzymatic hydrolysis of proteins results in smaller molecular mass, with less secondary structure and in some cases improved functional properties compared to the intact protein (Adler-Nissen & Olsen, 1979). The hydrolysates are physiologically better than intact proteins because their intestinal absorption appears to be more effective due to the increase of solubility (Ziegler *et al.*, 1998). Limited proteolysis or phosphorylation increases a protein's negative surface charge and thereby increases solubility and improves other functional properties such as emulsifying activity and emulsion stability (Campbell *et al.*, 1996).

Enzymatic protein hydrolysates have been reported as suitable sources of protein for human nutrition because of their gastrointestinal absorption, especially di- and tripeptides, which seem to be more effectively digested than either intact protein or free amino acids (Ziegler *et al.*, 1990; Siemensma *et al.*, 1993). So developing short chain soy peptides, especially di- and tripeptides products for infant, patient, and geriatric is of great benefit.

In the study of enzymatic hydrolysis and synthesis of soy protein to improve its amino acid composition and functional properties, Calderón de la Barca *et al.*

(2000) obtained fractions (FI-E > 10 kDa, 10 > FII-E > 3 kDa, and 3 > FIII-E > 1 kDa) of SPI hydrolysates using ultrafiltration and methionine enriching with perfect amino acid composition for infants and a hypothetical no allergenic responses, FIII-E could be used in hypoallergenic formulas as the only source of protein. FII-E meets the amino acids requirement for children and adults, and it could be used in fortified soluble formulas and as the only protein source in special medical diets. FI-E, because of its high methionine content (4.2%) and foam stability and emulsifying activity, presents a good source of protein for baby food.

Isoflavones are phytochemicals (phytoestrogens) exist in leguminous plants, especially in soybeans, and have been associated with health benefits, including the potential to reduce the risk of age-related and hormone-related diseases, such as cancer, menopausal symptoms, cardiovascular disease, and osteoporosis (Adlercreutz, 2002; Omoni & Aluko, 2005). Isoflavones present in soybeans and soy foods as two forms, aglycones (daidzein, genistein and glycitein) and their glucosides. Greater than 90% of the total isoflavones isomers in soymilk exist as glucosidic forms (Murphy *et al.*, 1999; Tsangalis *et al.*, 2002). In general, glucosides are the mainly exist forms of isoflavones in soybeans and rarely as aglycone forms unless they have been fermented (Wang & Murphy, 1994).

Researchers (Izumi *et al.*, 2000) observed that isoflavone aglycone forms were absorbed faster and in greater amounts than their glucoside forms in humans. Additionally, it was observed that isoflavone glucosides could not absorbed intact across the enterocyte of healthy adults, and the initial hydrolysis of the sugar moiety by intestinal β -glucosidases was required to increase their bioavailability (Setchell *et al.*, 2002). Thus it is probably more effective in preventing chronic disease by consuming isoflavone aglycone-rich soy foods. Transformation of bioinactive soy isoflavone glucosides to bioactive isoflavone aglycones is an effective method to improve the biological activity and nutritious value of soy protein. Commercial β -glucosidases have been used in conversion of isoflavone glucosides (Park *et al.*, 2002, 2003) and β -glucosidases from *bifidobacterium* have been applied to hydrolyse the β -1,6 glucoside bonds to produce bioactive isoflavone aglycones in soymilk (Tsangalis *et al.*, 2002, 2003).

Unexpectedly, it was observed that hydrolysing isoflavone glucosides to aglycones by β -glucosidase in a soy drink before consumption did not enhance the absorption of isoflavones in post-menopausal women (Richelle *et al.*, 2002). On the contrary, fermentation of cooked soybeans by *Rhizopus oligosporus* (tempeh) was observed to increase the bioavailability of isoflavones in men and women (Slavin *et al.*, 1998). Possibly by hydrolysing isoflavone glucosides into bioactive aglycones, intestinal

microflora plays an important role in the ultimate bioactivities of isoflavones (Hendrich, 2002; Turner *et al.*, 2003). Consequently, both the enrichment of isoflavone aglycone prior to consumption and the modulation of intestinal microflora via the ingestion of viable bacteria may be required to increase the bioavailability of isoflavones of soy foods (Tsangalis *et al.*, 2004).

Donkor & Shah (2008) investigated the effect of hydrolysis of isoflavone to aglycones in fermented soymilk by probiotic organisms *Lactobacillus acidophi-*

ilus LAFTI L10, *Bifidobacterium lactis* LAFTI B94, and *Lactobacillus casei* LAFTI L26 at 37 °C for 48 h and then stored for 28 days at 4 °C and observed that conversion of glucosides to aglycones. They found that all the bacteria produced β -glucosidase, which significantly hydrolysed isoflavone β -glycosides to isoflavone aglycones in the fermented soymilk. They indicated that it is likely to improve the biological functionality of soymilk by increased isoflavone aglycone content. A summary of biotransformation of isoflavone glycosides

Table 2 Summary of biotransformation of isoflavone glycosides to aglycones by β -glucosidase hydrolysis

Sources of hydrolysate	Enzymes	Conditions	Results	References
SPI	β -Glucosidase	Using 11 strains of probiotic bacteria in 4% (w/v) SPI dispersion at 37 °C for 72 h with 2% (w/v) lactose supplementation	Supplementation of lactose enhanced the biotransformation of isoflavone glucosides to isoflavone aglycones	Ding & Shah (2010)
SPI	β -Glucosidase	Using <i>Lactobacillus acidophilus</i> , <i>Bifidobacterium lactis</i> , and <i>Lactobacillus casei</i> to ferment 4% (w/v) SPI dispersion at 37 °C for 48 h and then stored at 4 °C for 28 days	Probiotic bacteria induced biotransformation of isoflavone glucosides to isoflavone aglycones, thus improves the biological functionality	Donkor & Shah (2008)
Soymilk	β -Glucosidase	Using <i>Lactobacillus paracasei</i> , <i>Lactobacillus acidophilus</i> , and <i>Bifidobacterium longum</i> at 37 °C for 24 h	Induced biotransformation of isoflavone glucosides to isoflavone aglycones, hence improves the biological functionality	Wei <i>et al.</i> (2007)
Soymilk	β -Glucosidase	Using <i>Lactobacillus paraplantarum</i> KM, <i>Enterococcus durans</i> KH, <i>Streptococcus Salivarius</i> HM and <i>Weissella confusa</i> JY to ferment soymilk at 37 °C for 12 h	Induced biotransformation of isoflavone glucosides to isoflavone aglycones, thus improves the biological functionality	Chun <i>et al.</i> (2007)
SPI	β -Glucosidase	Using six strains of <i>Lactobacillus acidophilus</i> , <i>Lactobacillus casei</i> and <i>Bifidobacterium</i> in 5% (w/v) SPI dispersion at 37 °C for 48 h	All six micro-organisms can hydrolyse isoflavone β -glucosides and among them <i>L. acidophilus</i> 4461 was the most efficient which produced 76.9% aglycone after 24 h of incubation	Otieno <i>et al.</i> (2006)
SPI	β -Glucosidase	Fifteen probiotic microorganisms were screened for β -glucosidase activity in 4% (w/v) soymilk during incubation for up to 48 h and storage at -80, 4, 24.8 and 37 °C for 8 weeks	<i>L. casei</i> strains showed the highest β -glucosidase activity after 24 h of incubation. During the 48 h fermentation, β -glucosidase from <i>Bifidobacterium animalis</i> BB12 showed the best stability and the stability were significantly higher at lower temperatures (-80 and 4 °C) than at higher temperatures (24.8 and 37 °C)	Otieno <i>et al.</i> (2005)
SPI and soy germ (SG)	β -Glucosidase	Three soymilk formulations (SPI/SG 9:1, 6:4 and 3:7, total of 4%, w/v) were fermented using <i>B. animalis</i> at 37 °C for 24 h	The highest concentration of aglycone was found in fermented SPI/SG 6:4, even though 74% of its isoflavone concentration was still in an isoflavone glucoside configuration	Tsangalis <i>et al.</i> (2004)
SPI	β -Glucosidase	Using four strains of <i>Bifidobacterium</i> in 4% (w/v) SPI dispersion supplemented with D-glucose and L-cysteine at 37 °C for up to 48 h	Supplementation of soymilk with D-glucose and L-cysteine could not enhance the hydrolysis of isoflavone glucosides to aglycones	Tsangalis <i>et al.</i> (2003)
SPI	β -Glucosidase	Using five strains of <i>Bifidobacterium</i> in 4% (w/v) SPI dispersion (soymilk) at 37 °C for up to 48 h	Four strains produced β -glucosidase. <i>B. animalis</i> , <i>B. longum</i> -a, and <i>B. pseudolongum</i> hydrolysed isoflavone malonyl-, acetyl- and β -glucosides to form aglycones, and transformed daidzein to equol in soymilk	Tsangalis <i>et al.</i> (2002)
Commercial soymilk	β -Glucosidase	Commercial soymilk was fermented using five strains of <i>Lactobacilli</i> at 37 °C for 24 h	The production of β -glucosidase by the lactic acid bacteria was strain-dependent: some strains could produce this enzyme while others could not	Choi <i>et al.</i> (2002)

to aglycones by β -glucosidase hydrolysis is presented in Table 2.

A number of biological activities [antioxidant, anti-thrombotic, angiotensin-converting enzyme (ACE) inhibition, antihypertensive and immunoregulatory properties] have been reported for soy peptides (Arnoldi *et al.*, 2001; Chen *et al.*, 2004; Gibbs *et al.*, 2004; Kong *et al.*, 2008) and antioxidant activity of soy protein hydrolysates has attracted researchers' interest and great advances have been made.

Several studies have been conducted to evaluate the antioxidant potential of soy protein hydrolysates as well as to the characterisation of the most active peptides (Chen *et al.*, 1998; Chiang *et al.*, 1999; Gibbs *et al.*, 2004), which contained 5–16 amino acid residues (Chen *et al.*, 1996). The antioxidant activity is affected by the amino acid sequence and the relationship of structure–activity has been established for soybean peptides, with one or more residues of His, Pro, Tyr and Trp and hydrophobic amino acids present (Chen *et al.*, 1995, 1996, 1998).

The antioxidant properties of soy protein hydrolysates have been attributed to the cooperative consequences of several properties (Chen *et al.*, 1998), including their ability to act as metal-ion chelator, to scavenge free radicals, hydrogen donor or oxygen quencher, and to the possibility of preventing the penetration of lipid oxidation initiators by forming a membrane around oil droplets (Peña-Ramos & Xiong, 2002). The processing conditions, the type of enzymes and the DH influence the antioxidant activity (Peña-Ramos & Xiong, 2002; Gibbs *et al.*, 2004). It was found that non-refined soy protein hydrolysates showed higher antioxidant activity than purified fractions (Chen *et al.*, 1995).

Moure *et al.* (2006) evaluated antioxidant properties of ultrafiltration-recovered soy protein fractions from industrial effluents and their hydrolysates and observed that fraction with molecular weight less than 10 kDa possessed the highest antioxidant activity. The hydrolysates demonstrated higher antioxidant activity and reducing power and enhanced radical scavenging capacity than the original fractions in emulsion. The hydrolysates with molecular weight between 30 and 50 kDa showed the highest hydroxyl radical scavenging capacity, and the highest Trolox Equivalent Antioxidant Capacity (TEAC) (up to 6.6 mM Trolox) with molecular weight higher than 50 kDa.

Kong *et al.* (2008) evaluated immunoregulatory properties of soy protein hydrolysates prepared with Alcalase and insoluble soy protein and observed that they were superior to other enzymes and soy proteins, had the highest immunomodulating activity on proliferation of murine splenic lymphocytes and phagocytic effect of peritoneal macrophages. They concluded that peptides from soy proteins with lower molecular weight and higher content of positively charge were effective in stimulating immunomodulating activity.

In general, soy protein hydrolysates exhibit promising prospect in special diet and nutritional effects.

Conclusions

Since soy protein is a nutritious while cheap protein source, development of better tasting soy foods or food additives is necessary. Based on the modern enzyme technology, not only scientific research, but also food industry has already made great progress on proteolytic hydrolysis of soy protein. Further research should be focused on improving products flavour. Accurate instruments and technology need to be developed to control and monitor DH. This will help control cleaving and exposing bitter amino acids at the surface of short peptide chains. Furthermore, new enzymes which eliminate bitterness or produce good aroma need to be developed. Multiple enzymatic hydrolysis such as various combinations of exo-peptidase and endo-peptidase could be used to reduce bitterness while increasing DH. Through modern enzymetechnology, more functional and nutritional food ingredients and healthy foods can be developed.

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