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# High pressure processing assisted enzymatic hydrolysis – An innovative approach for the reduction of soy immunoreactivity

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ABSTRACT

Soybean (*Glycine max* (L.) MERR.) is recognized as a potent food allergen causing one of the most frequent food allergies worldwide. The effect of high pressure processing (HPP) prior to and during enzymatic hydrolysis using the enzyme preparation Flavourzyme® on the degree of hydrolysis (DH), molecular weight distribution (SDS-PAGE) and β-conglycinin (Gly m5) immunoreactivity of soy protein isolate (SPI) was studied. Enzymatic hydrolysis was carried out at atmospheric pressure (0.1 MPa) and HPP (100–600 MPa) at 50 °C for 15 min. Pressures higher than 300 MPa enhanced the degradation of Gly m5, which was confirmed by SDS-PAGE and LC-MS/MS analyses. The immunoreactivity of the samples was assessed by in vitro sandwich ELISA using mouse monoclonal anti-Gly m5 antibodies. Depending on the antibody tested, the residual immunoreactivity was completely inhibited or significantly impaired up to 99.5% applying HPP during hydrolysis at 400 and 500 MPa. By means of principal component analysis, the *beany* and *green* off-flavors characteristic for unprocessed SPI could be reduced by pressure enhanced hydrolysis at 400–500 MPa. The resulting hydrolysates possessed improved protein solubility, foaming activities and oil-binding capacities, which were improved by 45%, 66%, and 210%, respectively. HPP prior to and during enzymatic hydrolysis at 400–500 MPa constitutes an innovative approach for the production of low-allergen food ingredients that combine good taste and enhanced functional properties.
*Industrial relevance:* Food allergy has emerged in the last years as the incidence and prevalence are rising dramatically. Up to now, enzymatic hydrolysis is the only feasible method to mitigate soy allergy. However, the major drawback associated with enzymatic hydrolysis is the incomplete destruction of allergenic epitopes and the formation of a strong bitter taste. This research activity demonstrates that high pressure assisted enzymatic hydrolysis using the enzyme preparation Flavourzyme effectively reduces the immunoreactivity of soy proteins. Degree of hydrolysis analysis, SDS-PAGE, mass spectrometry as well as sandwich ELISA with mouse monoclonal anti-Gly m5 antibodies have been applied to analyze the destruction of allergenic proteins as well as to determine the residual immunoreactivity. This study provides preliminary evidence that this innovative combination process of high pressure and enzymatic hydrolysis has great potential to produce tasty low-allergen soy-based food ingredients with good physicochemical properties, i.e. protein solubility and foamability.


## 1. Introduction

Due to the rising prevalence and incidence of food allergies, public awareness and concern have initiated intensified efforts to treat and mitigate allergic reactions. The World Allergy Organization (WAO) estimated that 250 million people worldwide suffer from some kind of food allergy, where infants are more affected (5–8%) than adults (1–2%) (Fiocchi et al., 2003). Food allergy is an immunoglobulin E (IgE)-mediated adverse immunological response of the immune system towards specific food components, which are triggered by antigenic proteins with molecular masses ranging from 7 to 71 kDa (Wilson, Blaschek, & de Mejia, 2005). The antigenic determinants of the proteins, which can be recognized by IgE antibodies (= allergenic epitopes) can be categorized into linear (continuous) and conformational (discontinuous) epitopes (Shriver & Yang, 2011).

Soybean (*Glycine max*) is an attractive ingredient for the production of a variety of foods due to its high functionality as well as nutritional

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value. However, soy belongs to the eight priority food allergens ("big 8") that are believed to be responsible for 90% of all IgE-mediated allergic reactions in the U.S. (Taylor & Hefle, 2001). The European Food Safety Authority (EFSA) reported that soy allergy prevails among 2.7% of the population. Up to now, eight allergenic soy proteins (Gly m1–Gly m8) have been registered by the International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-Committee (www.allergen.org), but only the two storage proteins β-conglycinin (Gly m5) and glycinin (Gly m6) have been related to severe allergic reactions (Holzhauser et al., 2009; Ito et al., 2011; Schiller et al., 2014).

Although symptoms of food allergies can be treated with medicine, including antihistamines and epinephrine, the only prophylactic approach hitherto to avoid an allergic response is the complete dietary exclusion of the offending food. So far, as food allergies emerged considerably in the last years, science and industry were searching for new thermal and nonthermal technologies to control food allergy, in particular soybean allergy (Shriver & Yang, 2011; Verhoeckx et al., 2015). Hypoallergenic foods currently available on the market are mainly produced using enzymatic hydrolysis, which has proven to be efficient to attenuate soy immunoreactivity (Wilson et al., 2005). Nevertheless, only complete hydrolysis up to amino acid level was shown to reduce the allergenicity to a level of safe consumption. The reason for mostly incomplete allergen destruction is the compact structure and folding of proteins, thus, epitopes are difficult to access or not susceptible for the proteolytic enzymes. In addition, enzymatic hydrolysis can have an adverse impact on food structure and organoleptic properties, liberating a strong *bitter* taste, which impedes the utilization as food ingredient (Seo, Lee, & Baek, 2008; Shriver & Yang, 2011).

Nonthermal processing technologies, in particular high pressure processing (HPP), have emerged in the field of food science in the last decade as effective food preservation alternatives to conventional heat treatment. The main application of HPP is the decontamination of foods, thereby extending shelf life, with little effects on flavor and nutritional value (Knorr et al., 1992; Oey, Lille, Van Loey, & Hendrickx, 2008; Tao, Sun, Hogan, & Kelly, 2014; Guerrero-Beltran et al., 2004). HPP is known to unfold proteins due to its substantial impact on tertiary and quaternary structure of proteins, mainly maintained by noncovalent (hydrogen, ionic and hydrophobic) interactions between amino acid side chains. While most applications of HPP are the inactivation of enzymes, i.e. lipoxygenase (LOX) (van der Ven, Matser, & van den Berg, 2005), there is evidence that HPP can reduce immunoreactivity of proteins and might stabilize or even activate enzymes (Dhakal et al., 2014; Eisenmenger & Reyes-De-Corcuera, 2009). Recent research focused on the effect of HPP on immunoreactivity, which is explained by different mechanisms, i.e. protein-denaturation and thus the induction of protein conformational changes, leading to unfolding of proteins into monomers, and allergen removal by extraction (Kato, Katayama, Matsubara, Omi, & Matsuda, 2000; Houska et al., 2009; Li, Zhu, Zhou, & Peng, 2012).

HPP combined with enzymatic hydrolysis may have a practical relevance as an attractive technology for modifying allergy-relevant epitopes by promoting hydrolysis due to enhanced accessibility to enzymes. However, few researchers have ascertained this process (Peñas, Prestamo, & Gomez, 2004; Garcia-Mora, Penas, Frias, Gomez, & Martinez-Villaluenga, 2015; Chicon, Belloque, Alonso, Martin-Alvarez, & Lopez-Fandino, 2008). In particular Peñas and colleagues confirmed a positive impact of HPP on hydrolysis of soybean whey proteins as they observed a reduced Gly m1 immunoreactivity combining HPP and hydrolysis using various food-grade enzymes (Peñas et al., 2004; Peñas et al., 2006b; Peñas, Prestamo, Polo, & Gomez, 2006a).

However, data about the effect of HPP combined with hydrolysis on the reduction of other potent soy allergens (Gly m5 and Gly m6) remain unstudied. As these two proteins are the most abundant storage proteins of soy and sensitization against these proteins is highly indicative for severe allergic reactions (Holzhauser et al., 2009), the evaluation of their residual immunoreactivity is indispensable for the assessment of the potential allergenicity of the modified food.

Therefore, the aim of this study was to determine the effect of HPP (100–600 MPa) prior to and during enzymatic hydrolysis on (i) the degradation of major soy allergen Gly m5 (SDS-PAGE and LC-MS/MS), and (ii) the residual immunoreactivity (sandwich ELISA) using mouse monoclonal anti-Gly m5 antibodies (mAbs). (iii) As the sensory and physicochemical properties of the resulting hydrolysates are of overriding importance for food industry, these characteristics have been analyzed. The food-grade Flavourzyme preparation was applied for enzymatic hydrolysis as this enzyme is - beyond the large variety of different proteolytic enzyme preparations - outstandingly effective by reducing or eradicating the bitter taste (Meinlschmidt, Scheiggert-Weisz, Brode, & Eisner, 2016a). A bitter taste is often perceived in protein hydrolysates due to the emergence of bitter peptides and might impair their application as food ingredients. However, Flavourzyme is not able to sufficiently degrade soy proteins at atmospheric pressure (Meinlschmidt, Sussmann, Schweiggert-Weisz, and Eisner (2016b) and the enzyme hydrolysis might be promoted by HPP treatment.

## 2. Materials and methods

### 2.1. Raw materials and chemicals

Untoasted soybeans (*Glycine max* (L.) Merr.) were purchased from Naturkost Ernst Weber (Munich, Germany). The enzyme preparation Flavourzyme® 1000 L (1000 LAPU/g, Leucine Amino Peptidase from *Aspergillus* (*A.*) *oryzae* was kindly provided by Novozymes A/S (Bagsvaerd, Denmark).

All chemicals used in this study were of analytical grade and obtained from Th. Geyer GmbH & Co. KG (Renningen, Germany) unless stated otherwise.

### 2.2. Preparation of soy protein isolates (SPI)

SPI was prepared from soybean seeds using the technique as described elsewhere (Meinlschmidt et al., 2016b). The SPI obtained had a protein content of 94.6% and a dry matter content of 94.4%.

### 2.3. High pressure (HP) equipment and high pressure processing (HPP) assisted enzymatic hydrolysis of SPI

#### 2.3.1. HP equipment

Pressurization was conducted in a laboratory system with indirect pressure generation (High Pressure Single Vessel Apparatus U4000, Institute of High Pressure Physics, Warsaw, Poland), a maximum operating pressure of 800 MPa, a volume of approximately 750 mL and a theoretically operable temperature range of −25 to 80 °C. This unit is composed of a biphasic pressure build-up. With the initial pump (piston) a pressure with 50 MPa less of the desired pressure is achieved up to 600 MPa. The fine tuning up to the desired pressure level is done in a second phase by another intensifier. Both intensifiers have a transformation ratio of 1:16. The pressure build-up up to 600 MPa took 5 s per 100 MPa (30 s from 0.1 to 600 MPa). A mixture of propane-1,2-diol (Sigma-Aldrich Corporation, St. Louis, Missouri) and deion. Water (1:1) was used as pressure transferring liquid (PTL) for the experiments.

#### 2.3.2. HPP assisted enzymatic hydrolysis

For all experiments described below, SPI dispersions containing a protein concentration of 5 g per 100 mL deion. water were prepared and adjusted from initially pH 6.7 to pH 8.0 with 1 M NaOH prior to treatment. Two sets of experiments were performed and are visualized in Fig. 1 (A: HPP prior to enzymatic hydrolysis; B: HPP during enzymatic hydrolysis).