Impact of Selected Processing Technologies on Immunoreactivity Reduction in Yellow Mustard *(Sinapis alba)* Allergens

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ABSTRACT

Food allergy is becoming a global challenge affecting daily life of many individuals all over the world. Mustard is included in the list of top eight priority food allergens and the important cultivars are brown mustard (*Brassica juncea*) and yellow mustard (*Sinapis alba*). Known allergens associated with food allergic reactions are Sin a 1, Sin a 2, Sin a 3, Sin a 4 in yellow mustard. Mustard is usually incorporated as ingredient in many products for flavoring, emulsification and water binding. It is necessary to understand not only the influence of processing techniques on the associated functional properties of the mustard proteins as well as its influence whether it results in increasing or mitigating allergenic potential of proteins. Processing technique such as thermal processing (heating), germination, fermentation etc., will vary in their influence on allergens.

In this study, the fate of mustard allergens through selected food processing technologies are investigated. First, thermal processing was explored for immunoreactivity reduction. Generally, it is recognized that thermal processing brings several changes in protein structure and renders them relatively less sensitive. Conventional cooking up to 60 min and intense thermal processing at 110°C to 120°C with duration between 15 and 120 min were applied to treat 5% mustard extract slurry samples and then sandwich ELISA assay was used to quantify residual immunoreactivity of allergic proteins. Enhanced thermal treatment resulted in allergen reduction by 99.9% whereas normal cooking only showed 67% reduction ($p < 0.05$). Fourier-transform infrared spectrometry (FTIR) results revealed secondary structure and conformational changes in mustard proteins. This suggested possible denaturation and/or unfolding which may have resulted in modifying immunoreactivity properties of mustard proteins. Quality analysis after intense thermal treatments showed minimal effect on color and viscosity attributes.

Selected novel and nonthermal food processing methods such as germination and fermentation were also investigated. Five-day germination at 35-40°C and fermentation for 3 days at 25°C and 35°C was carried out and allergen activity was quantified by enzyme-linked immunosorbent assay. Germination and fermentation resulted in about 85% reduction in immunoreactivity ($p < 0.05$). In addition, combination of both methods induced up to 90% decrease in immunoreactivity. Germination and fermentation could result unfolding, denaturation and aggregation depending on the degree of exposure of conformational epitopes within molecule structure. Conventional cooking of 5% slurry extract of germination or

fermention treated samples at 100ºC demonstrated further percentage reduction in immunoreactivity by ~98% while cooking alone was effective only to reduce it by 70%. In addtion, these treatments resulted increasing antioxidant capacities of yellow mustard seeds ($p < 0.05$).

In conclusion, these selected processing methods are promising, safer and practical way to significantly bring down the immunoreactivity of mustard allergens. This study is clear step forward in understanding different processing treatments for allergen immunoreactivity reduction and unique first-time study which might turn out to be a boon to the population that are allergic to mustard proteins.

RÉSUMÉ

L'allergie alimentaire est devenue un défi mondial affectant la vie quotidienne de nombreuses personnes autour du monde. La moutarde est incluse dans la liste des huit allergènes alimentaires prioritaires dont les cultivars importants sont la moutarde brune (Brassica juncea) et la moutarde jaune (Sinapis alba). Les allergènes connus associés aux réactions allergiques alimentaires sont Sin a 1, Sin a 2, Sin a 3, Sin a 4 dans la moutarde jaune. La moutarde est généralement incorporée comme ingrédient dans les sauces, les vinaigrettes ou les poudres aromatisantes et utilisée comme émulsifiant et agent liant d'eau. Il est nécessaire de comprendre l'influence sur la fonctionnalité propriétés des protéines lors de la transformation des aliments pour comprendre si une technique de transformation spécifique permet d'augmenter ou d'atténuer le potentiel allergène des protéines. Les techniques de traitement telle que le traitement thermique (chauffage), la germination, la fermentation, etc., variera dans son influence sur les allergènes.

Dans cette étude, le destin des allergènes de la moutarde selon plusieurs technologies de transformation des aliments est étudié. Au début, le traitement thermique a été étudié pour la réduction de l'immunoréactivité. Usuellement, c'est connu que le traitement thermique apporte plusieurs changements dans la structure des protéines et les rend relativement moins sensibles. Une cuisson conventionnelle jusqu'à 60 min, et un traitement thermique intense de 110°C à 120°C dans une durée entre 15 et 120 min ont été appliqués pour traiter les échantillons de suspension d'extrait de moutarde à 5%, puis un test ELISA a été utilisé pour quantifier l'immunoréactivité résiduelle des protéines allergiques. Une réduction des allergènes de 99,9 % a été réalisé avec le traitement thermique amélioré, alors que la cuisson normale n'a montré qu'une réduction de 67 % ($p < 0,05$). Les résultats de l'analyse par spectroscopie infrarouge à transformée de Fourier (FTIR) ont démontré une structure secondaire et des changements dans la conformation des protéines de moutarde. Ceux-ci suggèrent la possibilité du dénaturation et/ou un dépliement qui a entraîné une modification des propriétés immunoréactif des protéines de moutarde. L'analyse de la qualité après les traitements thermiques intenses a montré un effet minime sur les attributs de couleur et de viscosité.

Les nouvelles méthodes de transformation des aliments non thermiques sélectionnées, telles que la germination et la fermentation, ont également été étudiées. Une germination de cinq jours à 35-40°C et une fermentation de 3 jours à 25°C et 35°C ont été réalisées, en plus l'activité

d’allergène a été quantifiée par un dosage immuno-enzymatique. Individuellement, la germination et la fermentation ont entraîné une réduction d'environ 85 % de l'immunoréactivité ($p < 0,05$). En outre, la combinaison des deux méthodes a induit une diminution de l'immunoréactivité jusqu'à 90 %. Ces résultats peuvent être causer par le dépliement, la dénaturation et de l'agrégation selon que les épitopes conformationnels sont exposés ou cachés dans la structure de la molécule. La cuisson conventionnelle d'extraits de suspension à 5 % d'échantillons sélectionnés traités à 100 °C a démontré une réduction supplémentaire en pourcentage de l'immunoréactivité enregistrée d'environ 98 %, tandis qu’une réduction de 70 % a été observé par cuisson seul. L’analyse de l'effet des méthodes biologiques a permis l’augmentation des capacités anti-oxydantes des graines de moutarde jaune ($p<0,05$).

En conclusion, ces méthodes de traitement sélectionnées sont un moyen prometteur, sûr et pratique pour réduire considérablement l'immunoréactivité des allergènes de la moutarde. Cette étude est un pas en avant dans la compréhension des différents traitements pour la réduction de l'immunoréactivité des allergènes, et une étude unique qui pourrait être une aubaine pour la population allergique aux protéines de moutarde.

CONTRIBUTION OF AUTHORS

This thesis consists of five chapters, presented in the form of manuscripts and in accordance with the guidelines for thesis preparation provided by the faculty of Graduate and Postdoctoral studies. Chapter I consists of a general introduction section with a brief perspective of allergens of yellow mustard and how selected processing methods affect allergens. It also details the objectives of the current study. Chapter II provides a comprehensive review of literature on allergens, effect of health and effects of food processing. Chapter III and IV constitute the main body of the thesis and have been submitted for publications. Chapter V comprise of general conclusions, contributions to knowledge and recommendations for future work.

Simran Kaur Jawanda reviewed the literature, designed the experiments, conducted all experiments, analyzed data, and prepared manuscripts for journal submissions and publications. Dr. H.S. Ramaswamy guided the design, conduction of experiments and advice on direction and editing of manuscripts.

LIST OF PUBLICATIONS AND CONFERENCE PRESENTATIONS

Manuscripts (drafts and submitted):

1. Jawanda S.K., Sarhangpour R., H.S. Ramaswamy. 2021a. Tracking mustard allergens through cooking and enhanced thermal treatments of slurry using sandwich ELISA. Submitted to Journal of Food and Bioprocess Technology. *(Paper in review)*
2. Jawanda S.K., Sarhangpour R., H.S. Ramaswamy. 2021b. Tracking immunoreactivity of yellow mustard allergens through seed germination and lactic acid fermentation. Prepared for submission to Journal of Food Science and Technology.

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ABBREVIATIONS

%	Percent
t	Time
min	Minutes
s	Second
ºC	Degree Celsius
ANOVA	Analysis of Variance
FTIR	Fourier Transform Infrared
NA	Not applicable
RH	Relative humidity
IgE	Immunoglobulin E
IgG	Immunoglobulin G
PR	Pathogenesis-related proteins
TLPs	Thaumatin-like proteins
Log(10) Reduction	Logarithmic cycle reductions
LTPs	Lipid transfer proteins
(nsLTP)	Non-specific lipid transfer protein
NIAID	National Institute of Allergy and Infectious Diseases
Lg	β-lactoglobulin
ELISA	Enzyme-linked immunosorbent assay
WB	Western blot
DB	Dot blot
EAST	Enzyme-allergosorbent test
RAST	Radioallergosorbent test
RIE	Immunoelectrophoresis
BHR	Basophil histamine release
BAT	Basophil activation test
PCR	Polymerase chain reaction
HPP	High pressure processing
PEF	Pulsed electric field
OECD	Organisation for Economic Co-operation and Development

NOMENCLATURE

Fo	Process lethality
Co	Cook value
L*	Lightness
a*	Redness
b*	Yellowness
ΔE	Total color change
IR	Immunoreactivity
OD	Optical Density
β	Beta-sheet in protein secondary structure
α	Alpha-helices in protein secondary structure
DPPH	2,2-diphenyl-1-picrylhydrazyl
FC	Folin-Ciocalteu method
GAE	Gallic acid equivalent

CHAPTER I

1. Introduction

Food allergy is a term used to define an abnormal immunological responses or allergic reactions that occurs reproducibly on exposure to certain foods. Food allergic disorders occur after consumption of certain kind of foods, and it may result in acute, potentially life threatening reactions and also chronic debilitating diseases like *eosinophilic gastroenteropathies* and atopic dermatitis (Sicherer & Sampson, 2009). Food allergy is initiated by food proteins when antigen receptor cross-linked immunoglobulin E (IgE) antibodies are bound to high-affinity receptor on *basophils* or mast cells surface and it is not same as food sensitivity which may be resulted by a food component (for e.g. lactose intolerance) (Gould et al., 2003). The non-IgE- mediated food allergies are well known and acknowledged. These are important to understand and treat the adverse reaction to food (Nowak-Węgrzyn et al., 2015). Virtually, a food allergy can be stimulated by any food material but the major allergens are hen's eggs, cow's milk, peanuts, soy, wheat, tree nuts, shellfish and fish. Majority of food allergens share a number of characteristics in common such as relatively stable to acid, heat and proteases and being water- soluble glycoproteins that are of size 10-70 kDa (Jiménez-Saiz et al., 2015).

At the global level, food allergies are on a rise. In the recent years, food allergies are increasing in the western countries. Moreover, it has been reported that food allergies affects 8% of children and 5% of adults, approximately worldwide (Sicherer & Sampson, 2014). In Canada, the priority food allergens are peanuts, sesame seeds, eggs, soy, milk, tree nuts (almonds, cashews, Brazil nuts, hazelnuts, macadamia nuts, pine nuts, walnuts, pistachio nuts and pecans), fish, molluscs and crustaceans, wheat or triticale (hybrid of wheat and rye grains), sulphites and mustard (Anonymous, 2017). Recently, more cases of mustard allergy are reported. Patients allergic to mustard may experience oral, mild, allergy-like symptoms, but severe symptoms, such as systemic reactions are reported to occur frequently (Koppelman et al., 2007).

Mustard is a herbaceous flowering plant that belongs to the *Brassicaceae* family which also include Brussels sprouts, broccoli, cauliflower, cabbage, radish, turnip, and fodder crop (i.e. rape seed) (Rancé, 2003). The types of mustard seeds used in food processing are black (*Brassica nigra* or black mustard), brown (*Brassica juncea* or Indian mustard) and white (*Sinapis alba* or yellow mustard) (World Health Organization, 2010). Mustard is commonly used as a condiment and added as an ingredient in cooking, processing, and pre-packaged food as a

flavouring or seasoning agent, emulsifier, and water binding agent for texture control (Lee et al., 2008). Mustard seeds are available as whole, grounded to powdered form and/or processed into prepared mustard products such as mustard sauce (composed of mustard seeds, vinegar, salt, and some other spices and additives) in the market. It is a major ingredient in North American-style mustard sauce. Brown and white mustard seeds are blended in preparation of English-style mustards and are also main ingredients in Chinese and European-style mustards (Posada-Ayala et al., 2015). All three kinds of mustard seeds are available in North America region. Moreover, Canada is leading country in the international mustard seed market that accounts for about 35% of the world population and 50% of the global exports (World Health Organization, 2010).

Mustard is reported as a frequent cause of allergic reactions and symptoms ranging from oral allergy syndrome to immediate skin response as well as severe reactions in hyper-sensitive patients such as anaphylactic shock (Caballero et al., 2002). Presently, there is no successful treatment for mustard allergy and avoiding mustard containing products is the only way to avoid allergic reaction. The allergic response to allergenic proteins depends upon number of factors including allergic proteins properties, sensitivity of affected persons, effect of processing on food containing allergens and food components and their interactions (Sathe et al., 2005). The part of food protein that may result in allergic reaction can be due to chain of amino acids in either primary structure or may be a three dimensional motif of structure of protein that is linear and conformational epitopes respectively. The allergic protein may have single epitope or number of different epitopes. For IgE-cross linking, more than one epitope on allergen is required. Understanding of these epitopes is important develop ways to decrease or eliminate allergenicity of targeted allergens (Sathe et al., 2016).

Food and food ingredients are exposed to several food processing conditions and these can result in alteration of epitopes that may potentially affect allergenic properties of proteins. Food processing bring changes in protein conformation and can destroy the existing epitopes on proteins or can generate new ones (i.e. neoallergens) (Jiménez-Saiz et al., 2015). There are different kinds of food processing methods classified as thermal and non-thermal treatments and type of treatment may have different effect on epitopes. Thermal processing achieved by using dry heat (roasting, frying, ohmic heating, infrared heating) or by wet heating that is autoclaving, cooking, blanching, steaming and extrusion. Non-thermal processing methods are soaking, milling, high-pressure processing, germination, fermentation, irradiation (Vanga et al., 2017).

Processing can result in alteration of protein structure that is unmasking/ masking or destruction of allergenic epitopes which may enhance, reduce, or have no change allergenicity of offending food (Maleki, 2004; Sathe & Sharma, 2009).

1.1. Thesis Research Objectives

The main objective of this thesis was to investigate how different food processing technologies could influence the mustard protein and bring about reduction in the immunoreactivity of allergens. Emphasis was placed on selected processing technologies such as intense thermal processing (resulting in higher thermal severity than conventional thermal processing), and selected nonthermal processing such as germination and fermentation.

Specific objectives of this study have been set to the following:

I. **Tracking mustard allergens through conventional cooking and enhanced thermal treatments of slurry using sandwich ELISA:** (1) Track the reduction in yellow mustard allergens related IR using sandwich ELISA technique through mild (cooking in boiling water), moderate and enhanced commercial thermal processing conditions (110-120 ºC, 15-120 min) (2) Evaluate a possible relationship between IR of mustard allergen with lethality or cook value of treated mustard samples (3) Examine FTIR spectroscopy use to understand conformational changes induced by intense thermal treatment and its correlation with the observed immunoreactivity values, and finally (4) Investigate the effect of these enhanced thermal treatments on quality of mustard slurry especially the color and rheological properties.

II. **Tracking immunoreactivity of yellow mustard allergens through seed germination and lactic acid fermentation followed by cooking:** (1) Track the reduction in immunoreactivity of mustard proteins through the nonthermal process approach of seed germination and lactic acid fermentation of yellow mustard seeds (independently, and in combination, with and without added stove top finish cooking) using sandwich ELISA technique (2) Use FTIR spectroscopy to understand conformational changes induced by the biological methods (germination and fermentation) and its correlation with the

observed immunoreactivity values and finally (3) Investigate the effect of these processing treatments on the antioxidant capacities of yellow mustard seeds.

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PREFACE TO CHAPTER II

Chapter II aims to provide the "Literature Review" or the background information on food allergens with special reference to mustard allergens, mechanism, detection methods and food processing techniques used for their mitigation. Hence, this chapter is sectioned into several parts which will explain characteristics of food allergens, immune response, detection methods, effect of processing on allergens.

CHAPTER II
LITERATURE REVIEW

2.1. Characteristics of Food Allergens

2.1.1. Sources of Food Allergens

Many foods can cause allergies, among them some are more common than others. Almost 90% of all serious food allergies are related to proteins (allergens) and are recognized in eight major food groups: milk, soy, egg, wheat, peanuts, tree nuts, fish, and shellfish. The food allergens known to mankind are naturally occurring substances from plant, animal or fungal origin. There are over 400 proteins isolated from different food materials that are responsible for causing allergic signs and symptoms in human beings (Boyce et al., 2010). Allergens of plant origin are generally storage proteins and pathogenesis-related (PR) proteins (Breiteneder & Radauer, 2004). Glutenins and gliadins are seed storage proteins in cereal grains whereas in dicotyledonous seeds, 2S albumins, 7S vicilins, and 11S legumins are seed storage proteins. There are number of PR allergens commonly found in a wide variety of plant foods such as Bet v 1–related proteins, β-1,3-glucanases and thaumatin-like proteins (TLPs), non-specific lipid transfer proteins, chitinases (Jenkins et al., 2005). Most commonly known food allergen of animal origin are milk caseins (Broekaert et al., 2008), fish β-parvalbumins (Ma et al., 2008) and shellfish tropomyosins (Lopata & Lehrer, 2009).

2.1.2. Nature of Food Allergens

Most of the food allergens are water soluble glycoproteins having molecular weight ranging between 10 kDa to 70 kDa. They are found to be stable to heat, acid and proteolytic treatments (Sicherer & Sampson, 2010). But it is observed that these traits vary considerably because during processing allergenic proteins can undergo modifications (Sathe et al., 2002). The oligomeric allergens like vicilins or allergens having reoccurring sequence of peptide units (for e.g. tropomyosins) have high immunogenicity as a result of increased which is due to increased IgE epitope valency (Breiteneder & Mills, 2005). Similarly, proteins like 2S albumins and lipid transfer proteins (LTPs) have high number of intramolecular disulfide bonds which can contribute to allergenicity and demonstrate more stability from enzyme degradation and thermal denaturation (Hoffmann-Sommergruber & Mills, 2009). It is known that binding of ligands like

metal ions, lipids and steroids to allergic proteins (such as caseins, parvalbumins, lipid transfer proteins, β-lactoglobulin (Lg) and Bet v 1–related food proteins) can enhance stability of structure and increase resistance to proteolytic enzymes, mostly proteases require flexibility for their action on substrate proteins (Sathe et al., 2016). In case of ligands, they bind to specific proteins by a very unique pathway which includes a number of complex interactions. For example, caseins (αs1-, αs2-, and β-caseins) consist of phosphate groups such as phosphoserine or phosphothreonine residues are metal chelaters which includes calcium, therefore forming microstructures known as nanoclusters. The nanoclusters combine to form larger structures consisting of approximately thousand nanoclusters corresponding to casein micelles found in milk (Breiteneder & Mills, 2005).

Paravalbumin has 3EF-hand motifs, two of which has abilities to bind to calcium and third one is silent, but it forms covering cap of hydrophobic surface. In paravalbumin, notable changes in conformations and loss of conformation dependent IgE epitopes is caused due to loss of protein bound calcium (Bugajska-Schretter et al., 2000). Intake of antacid by persons allergic to parvalbumin is matter of concern as calcium ions will be removed to acid neutralization increasing parvalbumin resistance towards pepsin hydrolysis as well as increasing vulnerability of sensitive individuals (Untersmayr et al., 2003). Epitope is part of protein which can be recognized by the antibodies or T cells and is determinant of immunoreactivity. The antibody-binding epitopes can be classified into two categories that is linear and conformational epitopes (Scott et al., 1998). The linear epitope is comprised of a short peptide chain of 8-15 amino acids, while the conformational epitope consists a three dimensional motif (Aalberse, 2000). Conformational epitopes are considered less important in case of food allergies except for the oral allergy syndrome as they are very susceptible to gastrointestinal digestion and heat denaturation (Lin & Sampson, 2009).

2.1.3. Mustard as an Allergen

The major allergenic proteins of mustard seeds has been recognized and characterized. The major allergen in mustard is 2S albumin which is a storage protein of seed composed of one heavy chain and one light chain that is 88 and 39 amino acids respectively. These polypeptide chains are linked with two disulfide bridges (Menéndez-Arias et al., 1988). Allergens that are known to be related to food allergic reactions are Sin a 1, Sin a 2, Sin 2 3, Sin a 4, Bra j 1, Bra n

1. The major seed storage protein of *Sinapis alba* that is Sin a 1 allergen protein is also isolated from rapeseed, sesame seeds, walnuts, Brazil nuts and castor bean. It has resistance to the thermal degradation (Teuber et al., 1998). Sin a 1 is major allergenic protein of yellow mustard belonging to 2S seed storage albumin which is a compact molecule with molecular weight of 14.18 kDa. This protein is thermostable has resistance towards in-vitro digestion by trypsin and proteins degradation by proteolytic enzymes (Menéndez-Arias et al., 1988). Recently, another storage protein, 11S globulin Sin a 2 of 51 kDa has been identified as an important allergen (Palomares et al., 2005). A number of allergens derived from the non-storage seed proteins have been identified (Sin a 3 is a non specific Lipid Transfer Protein of 12.3 kDa and Sin a 4 is a profilin with molecular weight of 13-14 kDa) demonstrating IgE cross-reactivity with peach and melon fruits respectively (Sirvent et al., 2009). The major food allergens of *B.juncea* and *B.napus* species are Bra j 1 and Bra n 1 respectively are storage proteins of 2S albumin class and are abundant seed proteins (Puumalainen et al., 2006). The structure of principal allergen of *B.juncea* seed that is Bra j 1 is very close to Sin a 1 (de la Peña, 1991).

2.2. Immune Response to Food Allergens

2.2.1. Mechanism

The allergic protein crosses the mucosal barrier and enter the immune system inducing the immunological tolerance by generating T regulatory cells or anergy of antigen specific lymphocytes (Berin & Mayer, 2013). There are several factors that lead to development of oral tolerance such as genetics, age condition of mucosal barrier and gut microbiota, dosage and frequent exposure and physical properties of antigen (Sicherer & Sampson, 2010). Exposure to food proteins in various susceptible individuals may cause allergy sensitization not result in oral tolerance.

Figure 2.1 demonstrates the pathogenesis of IgE-mediated food allergy. When food proteins cross the mucosal barrier, then dendritic cells absorb these proteins and enters local nymph nodes. The allergic proteins are broken down to peptides and primary histocompatibility complex class II molecules introduced to native $CD4^+$ T cells on the dendritic cell surface. Then native T cells will be activated into T_H2 cells, resulting in production of cytokine [i.e. interleukin (IL) IL-4, IL-5, IL-9 and IL-13]. Eventually, naïve B cells are activated by T_H2 cells and IgE class-switching with IL-4 and IL-13 is induced. The activated B cells clones have a high antigen

affinity which can be further expanded with T_H2 cells. Reciprocally, Activated B cells had more T_H2 cells activated and act as antigen-presenting cells. Activated B-cells had produced IgE molecules which are bind to high affinity IgE receptors (FcεRI) on the basophils and mast cells. Degranulation process starts on re-exposure of allergen molecule to cross-link IgE molecules present on the granulocytes. Then, acute-phase reactions happen because of release of mediators (histamines, inflammatory lipids, chemokines and cytokines). Eventually, eosinophils and T_H2 cells assemble at reaction site due to chemokines produced by mast cells. T_H2 cells produce IL-5 and IL-9, activating eosinophils and mast cells and releasing more mediators and this establishes late-phase reactions (Larché et al., 2006).

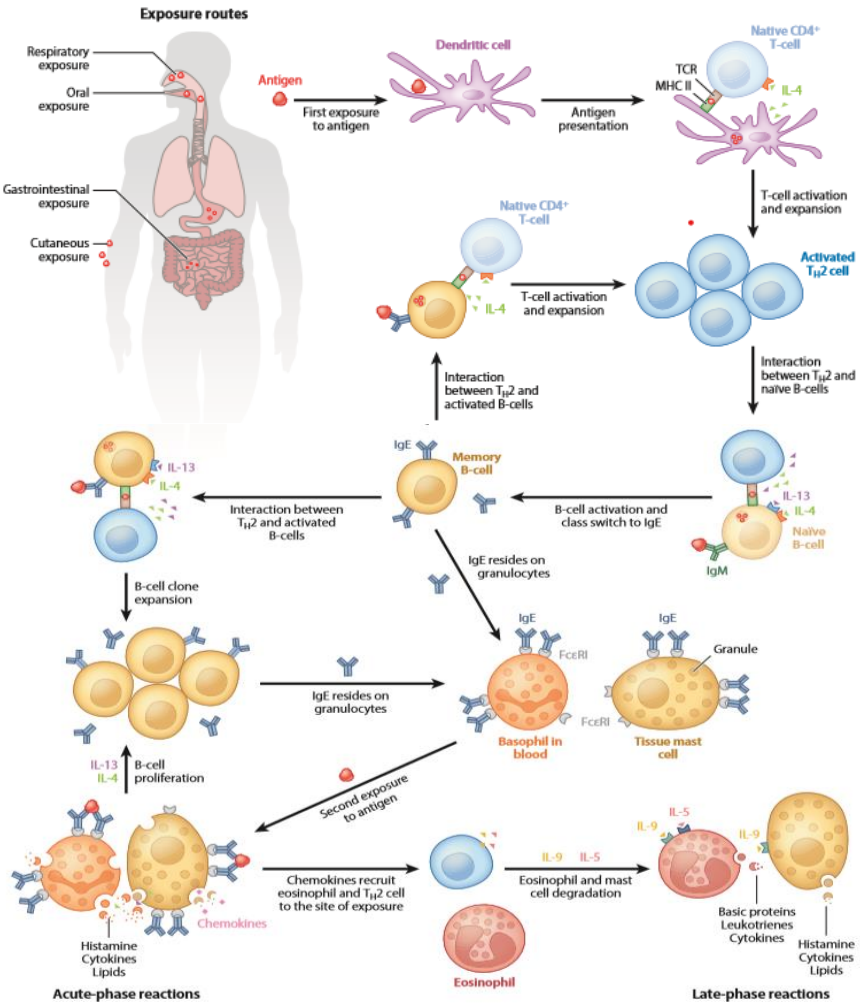

Figure 2.1 Illustration of pathogenesis of IgE-mediated food allergy (Source: Sathe et al., 2016)

2.2.2. Diagnostic Methods

National Institute of Allergy and Infectious Diseases (NIAID) sponsored an expert panel in 2010 in United States that had developed the guidelines on diagnosis and management of food allergies (Boyce et al., 2010). The panel recommended the use of following parameters to diagnose IgE-mediated food allergy which included recording medical history and physical examination, allergen-specific IgE test, a skin-prick test and oral food challenges. Various tests such as total serum IgE test, intradermal test, atopy patch test or combining specific IgE test, skin-prick test and atopy patch test, were not recommended by the panel. However, specific IgE and skin-prick tests are frequently used rapid diagnostic methods, but test results do not always match up with clinical symptoms, because the response levels continue down to very low levels which can go below the detection or quantifiable level of a testing method. Therefore, a double-blind placebo-controlled food challenge was recommended as best method for food allergy diagnosis but not used for routine purposes due to high cost, time and risk related to it (Pongracic et at., 2012). Now a days, several methods like component-resolved diagnostics, basophil activation, epitope-binding patterns metabolomics and T-cell proliferation are proposed as they improve testing efficiency along with decreasing cost and risks related to it (Sicherer & Sampson, 2014). Also today more sophisticated methods based on LC-MS approaches are also available and are being evaluated for such quantifications.

2.2.3. Symptoms and Treatments

The adverse reactions such as diarrhea, nausea, vomiting and abdominal pain for GI disorders; nasal congestion, cough, sneezing, wheezing and dyspnea for respiratory disorders; angioedema and pruritus for oral allergy syndromes and angioedema and urticaria for cutaneous disorders are observed on exposure to the offending food. The food-induced anaphylaxis and even deaths had been recorded (Sampson, 2004). When person is diagnosed with food-induced anaphylaxis, an intramuscular injection of epinephrine is administered followed by immediate transportation to hospital emergency facility (Boyce et al., 2010). For treating the food allergies, treatment like oral, sublingual, subcutaneous and intra-lymphatic immunotherapies are used before appearance of symptoms. Protein/peptide vaccines, plasmid DNA–encoded vaccines, anti-IgE antibodies, adjuvants, prebiotics, probiotics, cytokines/anticytokines and bacterial lysates are reported for immunotherapy (Sicherer & Sampson, 2014). More detailed studies and evaluations

are required to ensure safety and efficacy of these therapies and till then the best defence is to strictly avoid offensive food (Boyce et al., 2010).

2.3. Detection Methods for Allergens

The detection methods are important for consumer protection from food containing allergens (EFSA Panel on Dietetic Products & Allergies, 2014). Sensitivity of the detection method can vary depending on requirements (Kirsch et al., 2009). The detection methods may either indirectly target a marker for allergen presence or target food allergen directly. Method selection depends upon number of factors such as food processing history, kind of food matrix, availability of antibodies and primers and identified target allergen (Kirsch et al., 2009) and human allergy epitopes as well as characterization of food allergens (Monaci & Visconti, 2009). The analysis of allergenic proteins from food matrices can show many additional problems from chemical interactions between non-targeted allergic proteins and the detection antibody (Montserrat et al., 2015).

2.3.1. Immunoassays

These assays can detect and quantify the interactions between targeted analyte/antigen and a detection antibody. There are several methods that use IgG antibodies extracted in animals or IgE antibodies isolated from the allergic patient sera directly (Platteau et al., 2011). Generally, immunoassays formats employed are Enzyme-linked immunosorbent assay (ELISA), Dot Blot (DB) and Western Blot (WB). ELISA is presently used for routine screening and quantification of target antigen or analytes due to its simple, rapid, sensitive, and cost efficiency testing (Jayasena et al., 2015). This kind of immunoassay includes target antigen/ analyte selection, extraction protocols, availability of reference standards, quantification units and quantitative ranges used for the immunoreactivity (Jayasena et al., 2015). Food processing, source of antigen and kind of food matrices may affect the performance of ELISA testing (Cucu et al., 2013; Montserrat et al., 2015). Therefore, it is important to deteremine sensitivity, specificity, and robustness of assay for achieving optimal performance of assay. For qualitative testing, dipstick method is recently employed and this is a rapid and simple format providing portability and operational ease (van Hengel, 2007). Methods like Western blotting and Dot blotting are used for detecting membrane-immobilized proteins (Poms et al., 2004).

There are other kinds of immunoassays such as enzyme-allergo-sorbent test (EAST) and radioallergo-sorbent test (RAST) which make use of anti-human IgE antibodies for detection of IgE antibodies present in human sera and majorly used for clinical diagnosis and sometimes for qualitative and quantitative analysis (Kirsch et al., 2009). Another kind of assay is rocket immuno-electrophoresis (RIE) in which gel containing antibody is used for formation of antigen-antibody precipitates as a result of allergens migration through the gel (Poms et al., 2004).

2.3.2. Cell-Based Methods

By these methods, detection of cell mediators is done that are released on exposure to allergic proteins (Poms et al., 2004) but these methods have limited use because of reqiuremnt of human sera (Kirsch et al., 2009). For example, Basophil histamine release (BHR) assay detect histamine on release by released by basophils and in case of hexosaminidase assay, β-hexosaminidase is detected upon its release from mast cells. The basophil activation test (BAT) is another method which is more sensitive and specific than BHR assay, detecting mediators expressions (e.g. histamine) and basophil surface receptors.

2.3.3. DNA-Based Methods

Polymerase chain reaction (PCR) is the DNA-based method that is employed to detect amplifiable gene segments present and coding for targeted allergen. Such DNA methods come with a unavoidable limitation that is lack of correlation between gene and allergen expression (Cucu et al., 2013). Moreover, this method is prone to effects from processing conditions and food matrix that may subsequently effect extractability, amplification and DNA integrity (Platteau et al., 2011). The conventional PCR is economical, sensitive, simple and used for qualitative analysis. Real-time quantitative PCR had been developed for the routine allergen screening processes (Kirsch et al., 2009).

2.3.4. Direct Methods

These methods are based on mass spectrometry that are significantly advanced techniques in terms of specificity and sensitivity, having improved identification, characterization and determination of the food allergens (Faeste et al., 2011). Liquid chromatography and Liquid chromatography–mass spectrometry (LC/MS) are the techniques that can be used for detection of targeted peptide or protein (Cucu et al., 2013). According to the

study by Shefcheck & Musser (2004), LC/MS analysis has become significant detection tool for identification of allergens in food. There are number of factors that can influence target molecular properties such as target selectivity, sensitivity, specificity, quantification, and environmental factors are major challenges while selecting LC detector. LC/MS technique is recently gaining importance as direct detection method (Cucu et al., 2013; Faeste et al., 2011). Furthermore, techniques like LC–MS/MS has superior characteristics such as increased sensitivity, recovery, improved reproducibility and quantification range (Heick et al., 2011). There is a recent study done by (Posada-Ayala et al., 2015) for developing a very specific and sensitive method based on measuremnts of musatrd allergens detecting mustard traces in food products. This method can detect the mustard allergens with great reliability and accuracy. This method can help in creating a crucial tool for food industry and applied to all other types of allergens. Additionally, contribute towards the efforts of regulatory agencies to control the food allergens in food products and better serve the allergic population.

2.4. Food Processing & Food Allergens

2.4.1 Food-Processing Methods

A large number of foods and food ingredients contain one or more types of allergens and are consumed both in raw form or as a processed product and ingested either alone or combined in various forms. It is possible that allergic patients are sensitive to raw version of food but not sensitive towards processed forms and vice versa. Therefore, studying the significance of food processing in case of food allergy is very crucial (Venkatachalam et al., 2008). There are various factors that are to be considered while making a choice of food processing method such as purpose, environment, economics, equipment available and desired outcome (Sathe & Sharma, 2009).

Food processing methods can be classified as thermal and non-thermal methods. Thermal processing is usually carried out on foods to reduce microbiological contamination, enhance texture, digestibility or for detoxification. Heat treatment is given in two different temperature forms that are dry heat (roasting, frying, grilling, baking) and wet heat (blanching, autoclaving, boiling, steaming, canning) (Sathe et al., 2005). Ohmic and dielectric (MW/RF) heating are novel thermal processing techniques which are still in early stages of exploration and are promising technologies to reduce immunoreactivity of food allergens (Vanga et al., 2017).

Heating may produce different modifications in food proteins such as denaturation of proteins, aggregation by disulphide and non-covalent bonds, peptide bonds hydrolysis, and reactions with other molecules of food such as carbohydrates and lipids. But most crucial factor that needs consideration is what effect heating has on allergenicity of the allergic components present in certain foods. Heating can result in either reducing allergenicity that is loss of epitopes or enhancing it by exposure of epitopes or generation of new ones (nonallergens) (Wal, 2003).

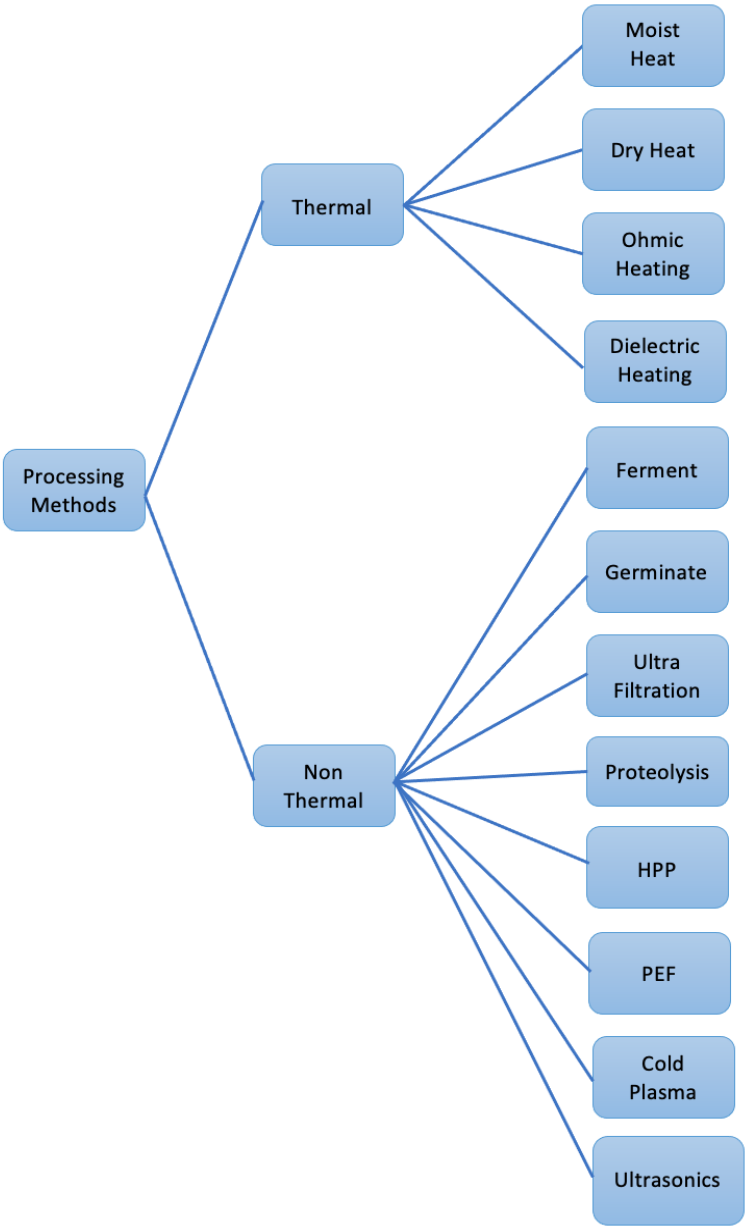

Figure 2.2 Different Types of Food Processing Methods

There are several non-thermal processing technologies like fermentation, germination, Ultra-filtration, proteolysis, high pressure processing (HPP), pulsed light, pulsed electric field (PEF), cold plasma, ultrasound. These non-thermal processing techniques have potential of inducing modification of protein structures and may mitigate the allergenicity of allergic proteins along with maintaining original characteristics of the food materials. Therefore, exploring the effect of non-thermal processing technologies on food allergens is becoming a topic of interest for researchers and showing new opportunities to produce hypoallergenic foods by using such approaches.

Recently, germination has gained a lot of popularity to produce hypoallergenic products. It is demonstrated that during germination, the seed storage proteins, including allergenic proteins, breakdown into peptides or amino acids by catalytic enzymes that are responsible for providing nitrogen required for seedling growth (Kang et al., 2007; Wu et al., 2012). Depending on specificity of enzymes and epitopes susceptibility to the active enzymes, germination may result in elimination of certain epitopes in seed storage proteins during germination period (Daussant et al., 1976). There are reported findings on rice and soybeans showing significant degradation of storage protein and reduction of immunoreactivity after short-term germination (Wu et al., 2012).

Fermentation is also considered a method to decrease immunoreactivity as demonstrated by studies done on fermenting soybean, skim milk and whey proteins (Chen et al., 2012). Lactic acid fermentation can decrease immunoreactivity of soya and it has potential of developing nutritious hypoallergenic soya products (Frias et al., 2008; Song et al., 2008). Ultrafiltration is another option in this direction and can be used when allergen sample has high thermal stability (Brenna et al., 2000). Using proteolysis as processing method can influence in vitro allergenicity of allergic proteins (Sen et al., 2002).

High pressure processing (HPP) is an excellent technology to produce low immunoreactive product and applied to allergens of food material like peanuts, sesame, soyabean, cowmilk to name a few (Chizoba Ekezie et al., 2018). The application of pulse light technology has been explored and efficient decrease in immunoreactivity of gluten observed by forming proteins with different molecular weight and rearrangement of protein structure. This was followed by unfolding of oligomeric and monomeric fractions of proteins and partial depolymerization (Panozzo et al., 2016). Cold plasma technology is gaining considerable

attention and research studies are needed to be conducted for attenuation of immunoreactivity of allergens (Chizoba Ekezie et al., 2018). Other non-thermal processing like ultrasound uses mechanical waves ranging between 20-100 kHz and shows positive effects of high intensity ultrasound on casein and whey proteins (Vanga et al., 2017). The application of one single processing technique may not show a significant reduction in immunoreactivity of allergic foods. The combination between various processing techniques can provide a new strategy for decreasing immunoreactivity (Dong et al., 2021).

All food processing approaches affect physicochemical properties of proteins in different ways and this influences allergenicity, bioavailability and gastrointestinal (GI) digestion. Heat absorbed through thermal processing can result in reducing allergenic potential by causing the changes in structure of proteins, alteration of IgE binding conformational epitopes and increasing digestibility along with having undesirable outcomes on the quality attributes of food product (Sathe & Sharma, 2009). On the other hand, the non-thermal methods may bring minimal changes in quality attributes of food and extending shelf-life of food. Non-thermal processing technologies are inexpensive, sustainable and eco-friendly technologies. Currently, hypoallergenic foods that are available in market are produced by enzymatic hydrolysis but negative effect on the organoleptic properties and this can make these products unacceptable to consumers. Along with processing methods, changes in the allergenicity is affected by various factors such as processing conditions, nature of allergen, effect of other food constituents, and binding interaction of allergen with antibodies (Vanga et al., 2017). For example, sesame proteins show different levels of tolerance after application of high-pressure treatments under varying conditions of pH and ionic strength (Achouri & Boye, 2013). Collectively, all these factors are also responsible for inconsistency and thus, making application of non-thermal treatments promising for mitigation of the food allergens. Moreover, a combination of different food processing methods may be more beneficial for the commercial applications. In reality, manufacturers have to incur additional cost for acquisition of novel techniques. In addition, studies showing any safety or toxicological risks as a result of modification of allergic proetins are relatively scarce, so all these processing methods may not be adopted until safety of using these methods is established (Chizoba Ekezie et al., 2018).

2.4.2. Effects of Food Processing on Allergens

Food processing may bring changes or modification in structure of proteins, interaction of food matric and/or solubility changes and this will finally affect the allergic potential of final product (Khuda et al., 2015). Protein can undergo changes such as unfolding, hydrolysis, polymerization, fragmentation, or aggregation. Depending upon the kind of treatment given and allergen present, allergenicity may decrease, increase or remain unchanged present (Sathe & Sharma, 2009). Allergen denaturation may result in destruction of conformational epitopes, exposure of masked epitopes or formation of new epitopes. This might explain the variability of allergenicity of the recorded allergenic foods. Usually, during food processing the conformational epitopes are more prone to undergo structural changes (Sathe et al., 2005). In the processing, when Maillard browning happen due to reaction between reducing sugar and amino group and this results in a process called protein glycation. Then, this can give rise to advanced glycation end products which may affect the allergenicity at the end. There are other kind of protein modifications that may occur such as deamination/deamidation reactions, oxidation, disulfide bond rearrangements, free radical damage, reactions with polyphenols (Davis et al., 2001). Depending on type of chemical changes will affect the action of proteins when released and digested by gastrointestinal (GI) tract and this may further affect the immune response of sensitive individuals (Mills & Mackie, 2008). Furthermore, there is a great need to study changes brought by a particular processing technology to decrease immunoreactivity of the allergenic proteins without compromising the quality attributes of affected food, tolerance threshold values for humans towards allergen and acceptable level of risk, which can be established and implemented by the regulatory agencies having authority to do so.

2.4.3. Food Processing & Mustard Allergens

It is observed that resistance to proteolytic digestion (stability), enzymatic changes of protein and glycosylation can affect immunogenicity and allergenic potential of particular protein (Huby et al., 2000). While considering food allergies, the effect of food processing and food matrix need to be taken into consideration while understanding allergic potential of proteins. Allergic reaction can be suppressed by food processing due to destruction of few epitopes but not completely eliminating allergenic potential of allergens. Allergenic properties are affected by the type of processing methods used for example roasting can decrease level of

allergenicity of most of food types but in case of peanut, it enhances the proetin allergenicity. Commonly used processing technologies are thermal processing, acid and enzymatic hydrolysis, change in pH, physical treatments (high pressure processing or extrusion) to name a few or combination of two or more of these techniques (Mills & Mackie, 2008). Presently, there are no effective treatments exist for mustard allergens. Therefore, sensitive individuals have to strictly avoid or eliminate mustard seeds and/or mustard-containing foods from their daily diet intake (Rancé, 2003).

However, there is need to study and use novel strategies including different types of thermal processing methods (boiling, autoclaving, roasting and acid digestion). Most of the mustard allergens are quite resistant to heat. For each protein, heat stability varies but it is always above 80°C. In a study, it was demonstrated that Bra j 1, a major allergen in oriental mustard, denatures at 82 °C (Jyothi et al., 2007). Interaction of protein with other constituents of food matrix like phenolic compounds can result in formation of more stable structure and this leads to increased requirement of temperature for initiating the protein denaturation process (Palomares et al., 2005).

The disulphide bonds contribute to form more stable and compact structure which may explain the heat stability of proteins (Schmidt et al., 2004). On the basis of study conducted by (Thomas et al., 2004), it was observed that during digestibility period of 60 min (pH 6.8 with pancreatin and pH 2 with pepsin respectively), proteins were resistant to gastric and intestinal degradation. They observed that digestibility of rapeseed proteins by pepsin and pancreatin was 83% in study of in vitro digestibility which is lower than what was observed for casein (97%). This is due to compact proteins due to high number of disulphide bounds resulting in low digestibility. This suggested that allergenic proteins of mustard remained intact throughout the applied food processing treatment (i.e. heating below 100ºC) and digestion. This could initiate an allergic reaction in allergic individuals (Savoie et al., 1988).

One of mustard species that is *Brassica napus* contains a 2S-albumin protein which is allergic seed storage protein, lack allergenicity records because of intensive mechanical and heat processing for oil production. Canola seed is traditionally crushed and solvent extraction is preformed to separate oil from the meal according to the data given by Organisation for Economic Co-operation and Development (OECD) in 2011. This process also includes other

steps also such as seed cleaning, pre-conditioning and flaking (i.e. preheating at 35 °C), seed conditioning (steam-heating with increasing temperature ranging between 80 and 105°C for 15–20 minutes), mechanically pressing to remove a portion of oil, removal of remainder of oil by solvent extraction of press-cake, oil and meal desolventisation (that is done with final stripping and drying at a temperature range of 103–107°C), degumming and refining of oil. This may be hypothesized that proteins removal and extraction of potential allergens can happen from the oil by extrusion process (Verhoeckx et al., 2015).

2.5. Conclusions

Allergenic protein structure may influence its biopotency. Food matrix components and their interactions can influence target allergenic protein which may further create complications for understanding the immune response of food allergen towards an affected individual. Moreover, on exposure to different types of food processing techniques, the native allergen structure may experience changes related to immunogenicity and immunoreactivity. A threshold or minimum dose can induce allergic reactions in a sensitive individual and the threshold values for most of the allergens are not yet established. Sensitive and robust methods for detecting and accurately quantifying targeted allergenic protein are required in a practical and economic manner for effective consumer protection.

It is already known that food processing bring changes in the allergenic capacities of food due to the alteration of epitopes of allergenic proteins. These proteins may undergo changes after processing which is directly related to higher or lower susceptibility to digestion. There is necessity to find the promising technologies that will prove to be successful in mitigating of food allergens.

Thermal processing at or below 100ºC and enzyme digestion are not enough for omitting allergenic potential of the mustard seed allergens. Therefore, exploration of different processing methods is required that might decrease immunogenicity and immunoreactivity of the mustard seed allergens. Intense thermal treatment may benefit in suppressing immunoreactivity of the mustard seed allergens. Furthermore, microbial fermentation and germination may also have the potential to decrease the allergenicity to such an extent that the reactions will not be elicited.

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PREFACE TO CHAPTER III

This study on yellow mustard allergens is first time study to understand the immunoreactivity reduction with different thermal treatment [temperatures (110ºC-120ºC) and times (15-120 min)] conditions specifically involving enhanced thermal processing. The focus of chapter III is to investigate relationship between enhanced thermal treatment and residual immunoresistance properties of a 5% mustard extract slurry. FTIR related information regarding wave number shifts and changes in secondary structure of mustard proteins after application of intense thermal processing were also to be gathered to get structural insight in to the treatments. Finally since these enhanced processing conditions can degrade the product quality, the effect of these treatments on quality attributes i.e., color and rheological properties of mustard extract slurry were also evaluated.

All the experimental work and data analysis were conducted by the candidate under the supervision of Dr. H. S. Ramaswamy.

Part of this study has been used for presentations and publications as follows:

Jawanda S.K., Sarhangpour R., H.S. Ramaswamy. 2021a. Tracking mustard allergens through cooking and enhanced thermal treatments of slurry using sandwich ELISA. Submitted to Journal of Food and Bioprocess Technology. *(Paper in review)*

Jawanda S.K., Ramaswamy H.S. (2021). Elisa based immune-reactivity assay to track the fate of mustard allergens through enhanced thermal treatments of mustard extract slurry, Institute of Food Technologists, (IFT), July 2021, Virtual on Zoom. (Poster to be presented; and virtual presentation for International Graduate Student Award)

Jawanda S.K., Vatankhah H., Sarhangpour R., Ramaswamy H.S. (2020). Impact of thermal processing conditions on fate of allergens in yellow mustard sauce. Northeast Agricultural and Biological Engineering Conference (NABEC), July 2020, The Pennsylvania State University, University Park, PA, USA. (Poster Presentation)

CHAPTER III

TRACKING MUSTARD ALLERGENS THROUGH COOKING AND ENHANCED THERMAL TREATMENTS OF SLURRY USING SANDWICH ELISA

3.1. Abstract

Thermal treatment is one of the most used methods for reduction of allergen in foods. Thermal processing brings several changes in protein structure and functionality and hence renders allergens to be relatively less sensitive. The objective of this study was to track yellow mustard allergens through the mild (conventional cooking) to moderate (conventional thermal processing) and enhanced thermal processing applications using immunoreactivity (IR) based sandwich ELISA technique. Using 5% (w/v) mustard slurry as test samples, cooking at normal temperature (100 ºC) was used up to 60 min used while conventional and enhanced thermal treatments were given at 110ºC to 120ºC with treatment times between 15 and 120 min. The cooking treatment reduced the allergen IR by 67% while the commercial and enhanced treatments clearly resulted in reducing the allergen IR level by over 99.9%. In logarithmic cycles, this translates ~0.5 vs. 3.5 log cycle reductions in allergen IR demonstrating multiple order of magnitude reduction enhancement in thermal processed samples. Conclusively, the residual IR detectable concentration of allergen was brought down from ppm to ppb levels. FTIR results demonstrated changes in mustard protein conformation as a result of denaturation and/or unfolding proteins causing reduction of allergen sensitivity mustard proteins. While allergen IR reduction was effective, the influence of enhanced thermal treatments on the color and viscosity of test samples was minimal. Therefore, these alternate enhanced thermal processing methods were considered to be very promising to bring down the IR of mustard allergens.

3.2. Introduction

Food allergy is becoming a global challenge that affects the daily life of many individuals. Traditionally, there are eight foods associated with most cases of food allergy and these are dairy, egg, fish, shellfish, peanut, tree nuts, wheat and soybean or their products (Hefle et al.,1996). Mustard is recently added to the list of priority allergens both in European Union and Canada (World Health Organization, 2010). Mustard is widely used in preparation of food products such as processed meats, pickled products, salad dressings, seasoning blends, sauces,

and condiments in order to enhance flavor or its nutritional value. Frequently, in products like sauces, salad dressings, or flavoring powders, mustard is included often as a hidden component. It is also used as an emulsifier and water-binding agent (Lee et al., 2008).

Mustard is reported as a frequent cause of allergic reactions and symptoms ranging from oral allergy syndrome to immediate skin response as well as severe reactions in hyper-sensitive patients such as anaphylactic shock (Caballero et al., 2002). Presently, there is no successful treatment for mustard allergy remediation and avoiding mustard containing products is the only way to avoid allergic reaction. There are 4 allergic proteins identified in yellow mustard seeds. Sin a 1 and Sin a 2 are major allergens and are characterized as seed storage proteins belonging to 2S albumin and 11S globulin family, respectively (Menendez-Arias et al., 1988l Palomares et al., 2007). Sin a 3 and Sin a 4 are a non-specific lipid transfer protein (nsLTP) and a profilin, respectively (Sirvent et al., 2009).

According to the literature, there are a number of reports revealing the ability of food allergens to be modified by food processing applications. Thermal processing can result in alteration of food protein structures causing unfolding, fragmentation, aggregation, hydrolysis or polymerization. Thermal processing treatment of food allergen can result in an increase or a decrease or has no influence (stabilize) on the allergenicity of proteins (Khuda et al., 2015; Sathe & Sharma, 2009). Modification of allergen depends on structure and chemical properties as well as the type of thermal treatment (dry or wet), temperature and duration of treatment, influence of constituents of food matrix and composition of food allergen (Mondoulet et al., 2005; Sanchez & Fremont, 2003). Each protein has a different level of heat sensitivity and, for example, Bra j 1 in brown mustard gets denatured at 82°C (Verhoeckx et al., 2015). Again, in general, thermal processing has been recognized to be effective in reducing immunoreactivity (IR) of the allergens, but under the most common thermal processing and cooking conditions the extent of IR reduction is rather limited, and often much below 90%.

Allergen detection and qualification is most commonly done by enzyme-linked immune-sorbent assay (ELISA) as it a very simple, sensitive, rapid and accurate method (van Hengel, 2007). Sandwich ELISA kits are used for identification and quantification of allergic proteins (Shim & Wanasundara, 2008). Moreover, protein structure changes are studied by Fourier transform infrared spectroscopy (FTIR). Amide I region of infrared spectrum are studied for protein secondary structure and frequently used for understanding the secondary structure

deviations such as conformational changes that is folding and/or unfolding of protein and aggregates formation (Carbonaro et al., 2012). This region is related to stretching of C=O of peptide backbone and N-H bending vibrations (Ahmed et al., 2017).

Currently, there is limited published work demonstrating the effect of food processing treatments on yellow mustard proteins. Moreover, the relationship between immunoreactivity of allergens in yellow mustard and heat severity has not been investigated yet. While published information on qualitative changes in allergen is quite abundant especially on denaturation and alteration in functional properties, studies based on quantifying the allergens in yellow mustard is almost nonexistent. Additionally, relation between IR and structural properties of mustard proteins has never been explored in details. Fourier transform infrared spectroscopy (FTIR) is a useful technique that can provide information on thermal processing effects on the secondary structure of mustard proteins which can be related to the IR changes of thermal treated mustard proteins.

Therefore, aim of this study was to (1) track the reduction in yellow mustard allergens related IR using sandwich ELISA technique through mild (cooking in boiling water), moderate and enhanced commercial thermal processing conditions (110-120 ºC, 15-120 min) (2) to evaluate a possible relationship between IR of mustard allergen with lethality or cook value of treated mustard samples (3) to examine FTIR spectroscopy use to understand conformational changes induced by intense thermal treatment and its correlation with the observed immunoreactivity values, and finally (4) to investigate the effect of these enhanced thermal treatments on quality of mustard slurry especially the color and rheological properties.

3.3. Materials and Methods

3.3.1. Preparation of Mustard Extract

Yellow mustard seeds (*Sinapis alba*) were purchased from Food to Live company (Brooklyn, NY). The experimental samples were prepared by grinding the mustard seeds and preparing a 5% (w/v) slurry with double distilled water. Then the slurry was filtered through Whatman #4 filter paper and the clear extract was collected. This mustard extract was heated to 100°C then cool down to 70°C (based on a preparation procedure for mustard sauce). It was then hot filled (85ºC) in to canning glass jars with 150 ml of sample, exhausted and sealed (Ramaswamy & Chen, 2004). Thus these products were subjected to some thermal pre-treatment

during sample preparations. Hence, the initial allergen protein level in the clear sample was lower than in the original.

3.3.2. Thermal Processing Treatments

Prepared mustard extract filled in to canning glass jars, 150 ml/jar, and capped (hermetical specially) were then processed (cooked) in an Instant Pot cooker (Instant pot Max programmable pressure cooker, 1100 W) at preset temperatures for different durations. This domestic cooker is designed to be used as a home canning equipment and had three settings provided cooking temperatures of 110, 115 and 120°C under pressurized conditions as is generally prevalent in commercial vertical retorts (Awuah et al., 2007). The operating procedure of the Instant pot cooker was similar to a batch steam based vertical retort processing. In order to provide steam, a small amount of water is added to the bottom of the cooker up to a check mark below the steam rack on which the glass jars were placed. The water was electrically heated. All treatments were performed in duplicates and conditions employed are detailed in Table 1.

3.3.3. Cooking of Samples

In addition, these samples were also cooked in boiling water at 100ºC for time intervals ranging between 10-60 min to evaluate the effect of extended cooking under atmospheric processing conditions and compare with those from the enhanced thermal treatments described in 3.1.

Table 3.1 Thermal processing and cooking treatments used for 5% mustard extract samples

Sample	Nominal Time	Nominal Temperature (°C)
1	60	110
2	90	110
3	120	110
4	30	115
5	60	115
6	90	115
7	15	120
8	30	120
9	45	120
Cooking	10-60	100

3.3.4. Enzyme Linked Immunosorbent Assay (ELISA)

A sandwich ELISA kit (3M Mustard Protein ELISA Kit, Maplewood, Minnesota, USA) was used for detection and quantitative analysis of mustard protein allergens. Sandwich ELISA uses of two matching antibody pairs (detection and capture antibodies). This ELISA kit contained a microtiter plate coated with an anti-mustard antibody, and reagents including mustard protein standard concentrate, mustard horseradish peroxidase (HRP) conjugate, an extraction buffer, diluent solution, wash solution, chromogenic substrate solution, and stop solution. Mustard proteins present in the test samples are extracted by the extraction buffer and it reacts with anti-mustard antibody that is adsorbed onto surface of polystyrene microtiter wells. Removal of unbounded fractions is done by washing with wash solution and anti-mustard antibodies conjugated with horseradish peroxidase (HRP) prepared with diluent solution are added. These enzymes, labelled antibodies, form complexes with previously bounded mustard proteins. This is followed by second washing step, and then the enzyme bound to immunosorbent is detected by adding a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB) and stop solution is added to stop reactions. Color is developed by the enzymatic reaction, and it varies directly with the concentration of absorbed mustard protein in sample tested. This procedure has been detailed in Lee et al. (2008). Then final absorbance of mustard protein in each test sample is recorded at 450 nm (iMark Microplate Absorbance Reader, Bio-Rad, Hercules, California, USA) as a measure of the concentration of mustard protein in the test sample. The concentration of mustard protein is calculated according to the standard curve obtained by the spiking different concentrations of supplied standards in the kit in the same manner and reading the absorbance (Okolie et al., 2018).

3.3.5. Process Lethality and Cook Value of Thermally Processed Samples

The intensity of cooking and enhanced thermal treatments was measured by using the concept of process lethality (Fo) and cook value (Co) as calculated by Eq. (1) & (2), respectively (Abbatemarco & Ramaswamy, 1993). For gathering temperature-time data, wireless temperature loggers were used and placed at the geometric centre of canning glass jar as well as in the cooking pot (Track Sense Pro, wireless loggers; Track Sense, Ellab Inc., Centennial, CO). Data were recorded using a data logger at 15 s intervals using the Agilent Data Acquisition System (HP34970A, Hewlett Packard, Loveland, CO).

$$\text{Process lethality, } Fo = \int 10^{((T-121.1)/z)}\, dt \qquad (1)$$

$$\text{Cook value, } Co = \int 10^{((T-100)/33)}\, dt \qquad (2)$$

where T and z represented temperature of treatment and z value, respectively.

3.3.6. Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) was used to study the changes in the secondary structure present in mustard protein. Test samples were first freeze-dried and a small quantity was weighed (0.1 g) and transferred on to the diamond crystal of the FTIR sample holder at room temperature. The data were gathered and analyzed by Windows-based OMNIC software (Version 8, thermo Nicolet Co., Madison, WI) using Nicolet™ iS50 FTIR Spectrometer with ATR accessory equipped with a computer. A total of 128 FTIR spectra scans at a resolution of 4 cm^{-1} were recorded and averaged in the mid-infrared region (4000-500 cm^{-1}). To avoid the influence of air, a background spectrum without the sample was collected before each determination. The spectra were deconvoluted and were used for calculating the secondary structure of mustard protein with a bandwidth of 31 cm^{-1} and an enhancement factor of 2.4 (Achouri & Boye, 2013). The results were prepared in terms of the percentage of secondary structures at corresponding wavenumbers.

3.3.7. Effect of Thermal Processing on Product Quality

3.3.7.1. Color Attributes

For color attributes, a Tristimulus Minolta Chroma Meter (Minolta Corp., NJ, USA) was used. The Chroma Meter was warmed up 20 min prior to use and the colorimeter was calibrated against a white blank standard (Alsalman & Ramaswamy, 2020). Ten measurements were made with each sample, and the values were averaged to obtain the L*(lightness), a* (green (−) to red (+), and b* (blue (−) to yellow (+)) values of the individual trails. The color difference (ΔE) was calculated using equation (3).

$$\Delta E = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \qquad (3)$$

where a_0*, b_0*, and L_0* represent initial value of untreated samples, while a*, b*, and L* represent values of thermally treated samples.

3.3.7.2. Rheological Characteristics

Rheological analysis was performed using a rheometer (AR 2000, TA Instruments, New Castle, DE, USA) supplied with a computer controlled rheology software with a cone and plate (60 mm diameter). Mustard extract (0.5 ml) was transferred on the bottom plate. The truncation gap size and temperature were set at 0.104 mm and 25°C, respectively. The flow tests were carried out by application of ramp up, hold and down shear rate regime going up from 0-100 s in 5 min, a 5 min hold at this maximum shear and a ramp down to zero in the next 5 min.

It was expected that the slurry would follow a power law model, but the clear nature of the filtered slurry and the resulting viscosity values were fairly low and hence an apparent viscosity index at the shear rate of 10 s^{-1} from the downward curve was taken as a measure of the sample rheology both before and after each treatment. Each sample was equilibrated in the plate for 3 min prior to the test (Vatankhah et al., 2018). Rheological data analysis was performed using a rheology advantage software (Rheology Advantage Data Analysis Program, TA, New Castle, DE, USA).

3.3.8. Statistical Analysis

The data were analyzed by one-way analysis of variance (ANOVA), followed by Post-hoc using Tukey's test ($p < 0.05$) using an SPSS 27.0 analytical software (SPSS Inc., Chicago, USA). All treatments and experimental studies were conducted in triplicates.

3.4. Results and Discussion

3.4.1. Allergen Concentration in Mustard Samples based on Immunoreactivity

ELISA results of the immunoreactivity of the cooked and thermally processed samples are presented in Table 3.2. The ELISA assay standard curve is relationship between OD values (absorbance) and standard (mustard allergen) concentration. The standard curve gives a somewhat nonlinear in a broad range of concentration tested but if taken in a narrower range give a good linear fit. In order to measure the allergen concentration level in the fresh sample which is

very high a suitable dilution is necessary before testing. The original samples were diluted several folds before spiking the microplates - generally 10^3 to 10^4 go get the absorbance range within the standard range. The mean value of mustard allergen in the slurry sample based on the ELISA IR testing was $26.9\times 10^3 \pm 0.38$ parts per billion (ppb) or 26.9 ppm.

Table 3.2 Quantification of residual mustard allergen IR in samples subjected to various cooking and thermal treatment based on ELISA assay

Sample	Nominal Temp (°C)	Nominal Time (min)	Mustard protein concentration (ppb)	Reduction in IR (%)	Reduction in IR [log(10)]	F_0 value (min)	C_0 value (min)
Control	-	-	$26.9 \times 10^3 \pm 0.38^{a1}$	-	-	-	-
Cooking	100	10	$25.0\times10^3 \pm 0.83^{ab1}$	07.09	0.03	0.08	10
	100	20	$22.2\times10^3 \pm 0.86^{b12}$	17.59	0.08	0.15	20
	100	30	$17.1\times10^3 \pm 3.01^{c23}$	36.66	0.20	0.23	30
	100	40	$12.7\times10^3 \pm 0.42^{d34}$	52.62	0.32	0.31	40
	100	50	$11.7\times10^3 \pm 1.72^{d34}$	56.42	0.36	0.39	50
	100	60	$8.83\times10^3 \pm 1.63^{d4}$	67.20	0.48	0.46	60
Thermal Processing	110	60	32.7 ± 0.95^{eA}	99.88	2.77	3.89 ± 0.14^{a}	120
	110	90	17.2 ± 0.43^{eCD}	99.94	3.19	5.38 ± 0.08^{b}	180
	110	120	07.2 ± 0.36^{eG}	99.97	3.57	7.20 ± 0.06^{c}	241
	115	30	21.3 ± 0.12^{eB}	99.92	3.10	8.54 ± 0.11^{d}	85.4
	115	60	18.2 ± 0.20^{eC}	99.93	3.17	14.7 ± 0.21^{e}	171
	115	90	15.1 ± 0.35^{eEF}	99.94	3.25	21.1 ± 0.15^{g}	256
	120	15	16.5 ± 0.83^{eDE}	99.94	3.21	9.34 ± 0.01^{d}	60.5
	120	30	15.1 ± 0.16^{eEF}	99.94	3.25	17.6 ± 0.06^{f}	121
	120	45	14.0 ± 0.56^{eF}	99.95	3.28	23.4 ± 1.09^{h}	181

Data are presented as mean ± SD of three independent observations. The mean difference is significant at the 0.05 level. Different superscript letters within a column indicate significant differences ($p < 0.05$).

The protein content of mustard seed is about 28% and that of mustard flour is about 40% (Bos et al., 2007). So, based on the mustard flour, the mustard protein concentration is about 2% (~20,000 ppm). However, since the sample was not a smooth homogenate, it had to be filtered to prevent variability in rest results which removed most of the solid particles and it further reduced the initial mustard allergen load in the sample. Further, the sample had to be subjected to several heat treatment steps during the preparation and filling, exhausting, holding between processing

steps (all these are operations taking place when the sample is at elevated temperatures between 70 and 100ºC) which result in a further lowering of residual allergen level in the control sample. However, for testing the influence of process variables on reduction in IR, this should not matter since the clarified slurry was used as the test sample both for control and testing.

3.4.2. Allergen Reduction Through Cooking

Table 3.2 also lists the allergen IR reduction in cooked samples which helped to reduce it by a margin of up to 67% down to about 8.8 ppm. On a logarithmic scale this resulted in less than one logarithmic cycle reduction. There was a steady decline in the allergen IR with an increasing cooking time. The cooking time depends on the product while a low range of 10-15 min could be sufficient for vegetables, 30-40 min may be required for cooking rice and perhaps more than an hour to cook large portions of food samples. Heat again may be supplied by boiling conditions in water, or steam cooking or baking, frying, broiling and other applications and the results obviously can be expected to be different. All these different types of thermal processing such as autoclaving, blanching, boiling, frying or roasting, will bring changes in protein structure, potentially changes in immunoreactivity responses (Maleki & Sathe, 2006). The cooking severity is directly measured in cook times for small samples (as used in this study) while the larger samples show much slower temperature rise in baking and other applications. In terms of equivalency to thermal processing severity which is measured in terms of a process lethality value which is a measure of the equivalent heating time at a reference temperature of 121.1ºC (250ºF), the cooking condition represents a very small fraction ranging from 0.1 to 0.5 min insufficient cause any significant destruction of microbial spore forming bacteria. So, in general, within the range of time tested, only minimal reduction was possible which would be insufficient in a majority of situations.

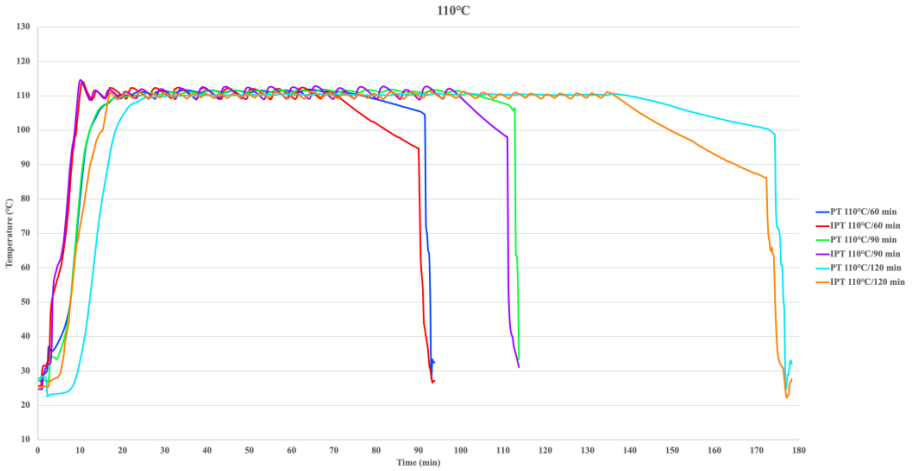
3.4.3. Allergen Reduction Through Thermal Processing

The purpose of commercial thermal processing (canning) is to kill or reduce the pathogenic anaerobic spore-forming bacteria (the target is *Clostridum botulinm* in low acid foods) to a statistically low levels (generally at least 12 logarithmic cycle reduction). This is generally taken as an equivalent heating time (Fo value, process lethality) of 3.0 min at 121.1ºC since the decimal reduction time of *C. botulinum* is taken as 0.25 min at the reference temperature. This is

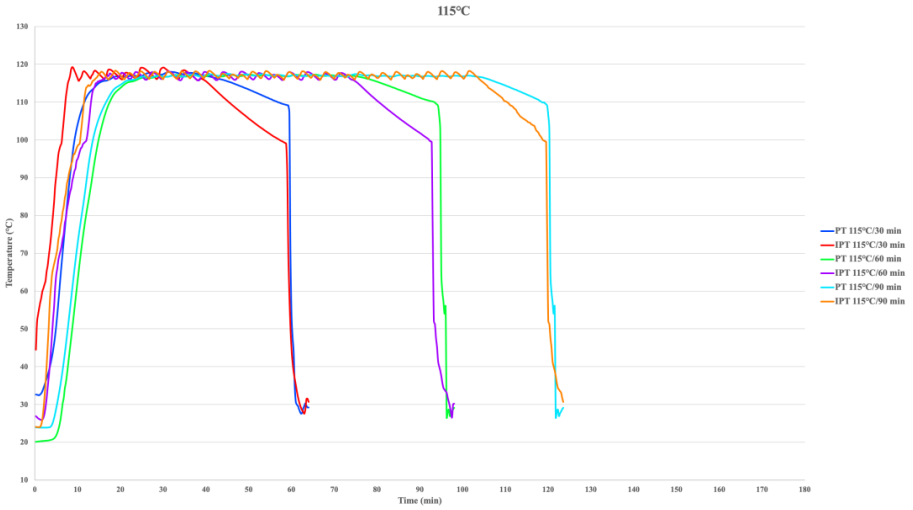
generally considered the minimum required for achieving commercial sterility of canned products which also requires vacuum packaging (which creates an oxygen free anaerobic atmosphere to protect product quality and inhibit aerobic microorganisms) and end product storage at 30ºC or less (to prevent growth of thermophilic bacteria). Further, although such a process will be safe from pathogen point of view, there could be other more resistant spore forming bacteria that could survive and spoil the product. Hence to have a better spoilage control, Fo values of 5-10 min are often employed in commercial canning applications. The immunoreactivity results shown in Table 3.2 demonstrate that reduction in immunoreactivity was the highest for the intense thermal treatment. These processes were given after determining the heat penetration profile of the product undergoing the process (Ramaswamy and Chen, 2004).

3.4.4. Temperature Profile during Testing

Time temperature profiles gathered during selected test runs are shown in Figure 3.1. and are grouped by processing temperatures. Each frame demonstrates the pattern for a given temperature processing conditions in which the Instant Pot temperature (steam medium) and the temperature of the product inside the glass jar are shown for three test runs each with different cook times. The curves demonstrate a come-up profile for the cooker, followed by a plateau portion represents the cook time until the cooling starts and then a part of the cooling is carried out slowly under pressure (pressure cooling) followed by pressure release and rapid cooling. The product temperature is also shown which lags a little bit behind the steam temperature in the pot. Product heating is quite rapid because the product is a liquid which is heated rapidly due to convection heating. These common trends can be observed and found to be rather smooth under all testing conditions. The data pertaining to these curves are discussed next.



a



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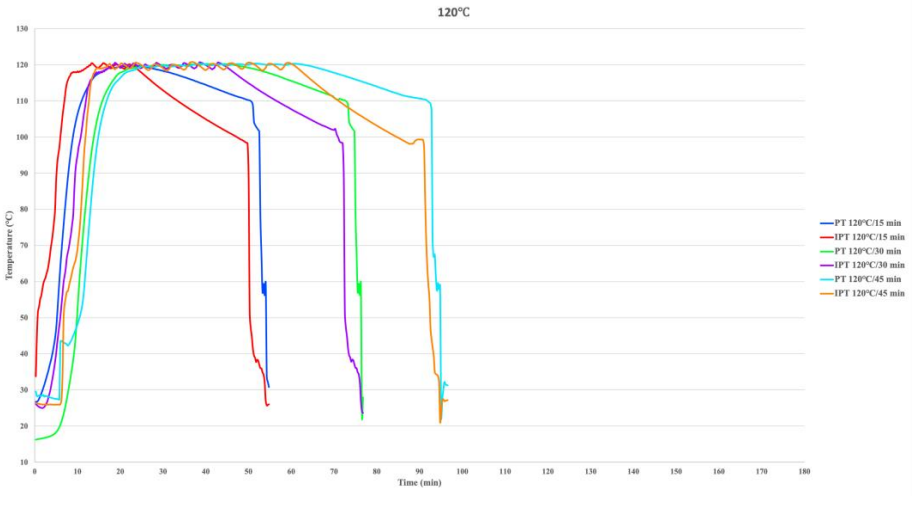


c

Figure 3.1 Time temperature profile of intense thermal processing done at different time-temperature combinations (a) 110ºC, (b) 115 ºC, (c) 120ºC

3.4.5. Heat Penetration Characteristics

The come-up time, process (cook) time and cooking (process) temperature data for the samples treated for enhanced thermal processing conditions (Table 3.2.) using Instant Pot cooker are summarized in Table 3.3. The come-up time varied depending on the cooking and room temperatures conditions and ranged from 13 -19 min after which the cooking is initiated. The average cook times achieved during the test runs were nearly the same as the original nominal times selected. The average process temperature (excluding come-up time) as measured was also nearly the same as the set temperatures as can be seen from the Table 3.3. These data indicated that the Instant Pot cooker performed well to create the intended processing test conditions.

Table 3.3 Heating conditions nominally employed and measured during the testing

Nominal Temperature (°C)	Nominal Time (min)	Come Up Time (min)	Processing Time (min)	Real Average Temperature (°C)
110	60	14.75 ± 0.71^{a}	59.88 ± 0.17^{c}	109.6 ± 0.28^{a}
	90	16.12 ± 0.53^{a}	90.65 ± 0.18^{b}	110.1 ± 0.15^{a}
	120	16.75 ± 0.35^{a}	119.9 ± 0.18^{a}	110.3 ± 0.14^{a}
115	30	13.12 ± 0.53^{a}	30.15 ± 0.53^{e}	114.9 ± 0.42^{b}
	60	15.62 ± 1.24^{a}	59.82 ± 0.25^{c}	115.12 ± 0.08^{b}
	90	16.56 ± 0.62^{a}	90.12 ± 0.53^{b}	115.1 ± 0.32^{b}
120	15	15.00 ± 1.06^{a}	15.12 ± 0.53^{f}	119.81 ± 0.03^{c}
	30	16.21 ± 2.17^{a}	30.5 ± 1.06^{e}	119.02 ± 0.12^{c}
	45	16.87 ± 0.53^{a}	45.20 ± 0.07^{d}	119.13 ± 0.42^{c}

Data are presented as mean ± SD of three independent observations. The mean difference is significant at the 0.05 level. Different superscript letters within a column indicate significant differences ($p < 0.05$).

3.4.6. Accumulated Process Lethality and Cook Values

The lethality and cook values computed from the time temperature data shown in Figure 1 are also shown in Table 3.2. Lethality values ranged between 4 to 23 min. The lethality at 110ºC which was in the range from 3 min to 7 min represented normal sterility conditions (~Fo = 5 min) which are commonly employed in commercial canning applications. Anything in excess

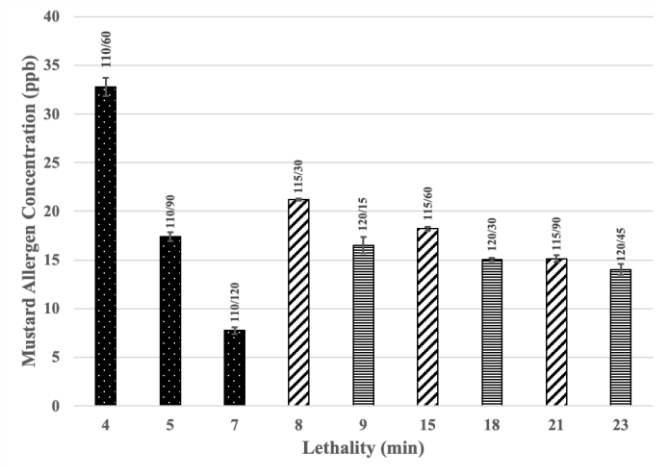
could be considered as enhanced thermal processing conditions which are unnecessary in the normal sense because they represent over-processing and unnecessarily lead to excess quality destruction. These are intentionally employed in the present study to evaluate their effects on allergen reduction fully knowing they might also possibly result in enhanced destruction of product quality. There might always exist certain types of products which can withstand such processes without severely impairing product quality. If they can be used by people who are sensitive to mustard allergens, they might benefit from such a process despite some loss in quality.

Further, the equivalent cook values were also computed for the different processing conditions based on a z value of 33ºC. These ranged from 10-60 min for the normal cooling and 60-260 min for the enhanced thermal processing conditions.

3.4.7. Influence of Enhanced Thermal Processing on Allergen IR Reduction

Figure 3.2 shows a bar graph (X-axis not to scale) of the ELISA IR response of mustard allergen retention as influenced by lethality (a) and cook value (b). In both cases, the general trend was that each temperature, a higher reduction in mustard allergen was observed with an increase in process times which elevated the associated process lethality or cook value. The trend with 110 ºC is distinct and well separated clearly showing a direct relationship of allregen IR reduction with increasing lethality or cook value. But the IR reduction achieved at 110 ºC / 120 min treatment is lower than those resulting with Fo of all higher lethalities at higher temperatures. Similar overlaps also can be seen with 115 and 120 ºC test runs. For example, the 9 min lethality from 120 ºC / 15 min and 115 ºC / 30 min treatments resulted in different allergen reduction and the further higher lethality did not cause much reduction.

In the range of processing conditions employed in the study, the 110 ºC for 60 min represented the minimal process that is required while 90-120 min represented little more severe conventional processing along with perhaps the 115 ºC for 30 min and 120 ºC for 15 min. In other words, within each of the three processing temperatures the lethality levels varied to include representation from conventional minimal, normal to some enhanced severity levels to test their ability to reduce mustard allergen IR.



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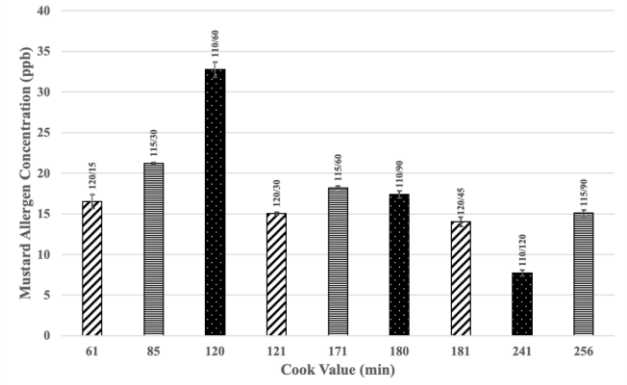


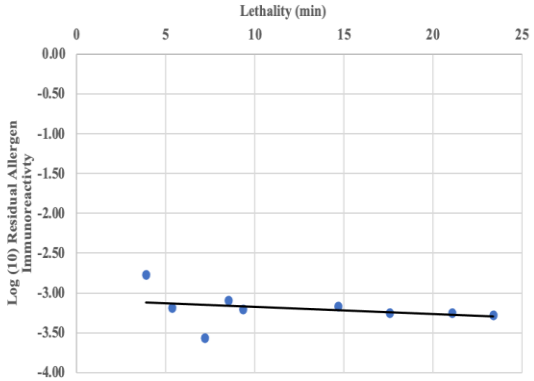
b

Figure 3.2 Elisa response of mustard extract with an increase in lethality (a) and increase in cook value (b)

Table 3.2 also summarized the allergen IR reduction achieved during these thermal processing treatments. The immunoreactivity reduction in mustard extract was lowest at 110 ºC with a 60 min process which is the minimal Fo for commercial processing applications. On the other hand, it was maximum at 110°C for 120 min which from heat severity point of view was not the most severe (Fo value only 7.9 min) but, time wise, it represented the longest duration. The lowest reduction value was 98.88% and the highest the reduction was 99.98%. In terms of logarithmic values this represented 2.77- 3.57 log (10) cycle reductions. The reduction at other two temperatures ranged between these two again representing lower value at shorter process times and higher at longer. Generally, severe thermal processing can lead to an increase in

protein denaturation and eventually resulting in aggregation and loss of functionality. The extent of effect of treatment depends upon both severity and duration of processing.

Results from Table 3.2 presented some very interesting observations. First, the conventional cooking conditions even up to an extended period of 60 min in boiling water resulted in only about 67% reduction in allergen immunoreactivity. With the allergen in the samples before treatment at high 27000 ppb levels, the cooking treatment reduced the value to around 9000 ppb level, still extremely high from reactivity point of view. In general, when the allergen level is in the low ppb levels, the product is considered to be potentially hypoallergenic. The conventional thermal processing treatment and the enhanced thermal treatments reduced the allergen levels to phenomenally low levels bringing the residual allergen concentrations to ppb levels (7-45 ppb). The best IR reduction was with a sample that had a residual 7 ppb of allergen, quite very low in terms of allergen threshold. Thus, the treatments would have a tremendous potential to reduce the allergenicity of mustard proteins.



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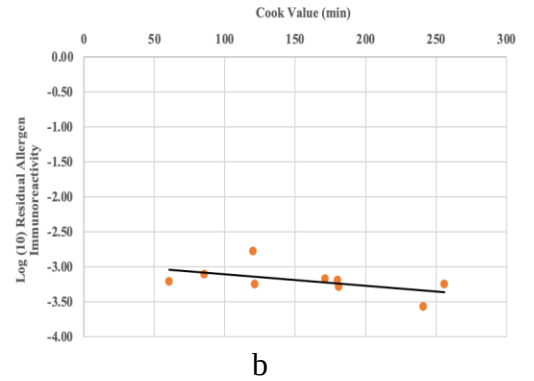

b

Figure 3.3 Effect of lethality (a) and cook value (b) on reduction of soy immunoreactivity in semi-logarithmic formats

Somewhat similar results were observed with cook values. With cook values beyond 60 min obtained under commercial processing conditions, the IR reduction of allergens reduced to below 25 ppb levels, except for 110 °C / 60 min with a 35 ppb residual IR allergen activity. Figure 3.3 shows the combined lethality and cook value influence on IR reduction which represented a semi-logarithmic relationship within the range of conditions covered by the thermal processing treatments. The overlaps which were mentioned are responsible for the spread in these plots, but in general they indicate a gradual reduction in the IR sensitivity with increasing lethality and cook values. These results show that both time and temperature are important in achieving the desired IR reduction in mustard allergens.

3.4.8. Effect of Process Time and Temperature on Allergen IR

Time effect at different temperature was included in Table 3.2 and discussed earlier with increasing treatment time resulting in higher IR reduction at any given temperature. Rearranged data in Table 3.4 indicates the temperature effect at selected treatment times in thermal processing situations. At any given time, the higher temperatures resulted in a greater effect on allergen reduction which is also related to higher lethality.

Table 3.4 Comparison of reduction in immunoreactivity of mustard protein treated at different temperature for same duration of time

Time	Temperature (°C)	Total mustard protein concentration (ppb)	F_0 Value
30	115	21.3 ± 0.12^{b}	8.54 ± 0.11^{c}
	120	15.1 ± 0.16^{d}	17.6 ± 0.06^{e}
60	110	32.7 ± 0.95^{a}	3.89 ± 0.14^{a}
	115	18.2 ± 0.20^{c}	14.7 ± 0.21^{d}
90	110	17.2 ± 0.43^{c}	5.38 ± 0.08^{b}
	115	15.1 ± 0.35^{d}	21.1 ± 0.15^{f}

Data are presented as mean ± SD of three independent observations. The mean difference is significant at the 0.05 level. Different superscript letters within a column indicate significant differences ($p < 0.05$).

3.4.9. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was used to investigate the effects of intense thermal processing on amide I and amide II region. In FTIR spectra, the amide I (1700-1600 cm^{-1}) and amide II (1575-1480 cm^{-1}) regions are most valuable for studying prominent vibrational bands in the protein backbone (Huson et al., 2011). The protein secondary structure is based on amide I region of the infrared spectrum and frequently used for studying the secondary structure deviations such as conformational changes that is folding and/or unfolding of protein and formation of aggregates (Carbonaro et al., 2012). This region is related to C=O stretching of peptide backbone and N-H bending vibrations (Ahmed et al., 2017). Amide I region include overlapping bands of various secondary structures that is β-sheets, α-helices, turns, and randomly coiled conformations (Wang et al., 2018). Deconvolution of the bands resulted in isolation of each band and distinguishing its frequency to assign it to the right secondary structure component and quantify it (Long et al., 2015; Martínez-Velasco et al., 2018). The assignment of amide I bands are β-sheets (1613-1637; 1682-1696), α- helices (1645-1662), turns (1662-1682, 1630), and unordered (1637-1645) (Dong et al., 2020). The typical FTIR spectra of amide I and amide II of unprocessed and processed samples were shown in Figure 3.4.

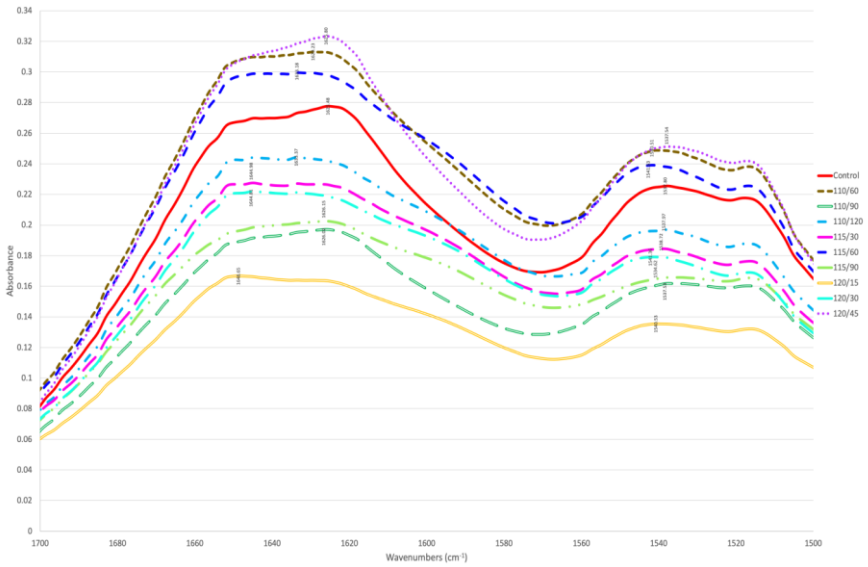

Figure 3.4 FTIR-spectra of Amide I and II components of unprocessed and thermally processed samples

The FTIR spectra of thermally processed samples showed significant changes in protein conformation based on the time and temperature combination of treatment applied. The FTIR

spectra recorded for treated samples at 120°C for 15 min, 120°C for 30 min and 115°C for 30 min showed a shift towards higher wavenumber as compared to other samples, with formation of more defined peak with band range intensities of 1640-1660 cm^{-1}, which represents the overlap of absorptions of helix and random coil structures. Similar results were reported by (Achouri & Boye, 2013), the FTIR spectra of thermally treated samples demonstrated significant changes in conformation of proteins depending of kind of treatment applied. In this study, it was observed that shift in bands were observed, with the transition of α- helices towards mores unordered structure. The wavenumber shifts were also observed in the band regions of 1560-1520 cm^{-1}. The amide II region represents N-H bending vibrations coupling with C-N stretching. It can be used for assessing protein conformations. Although, it originates from the complex vibrations including multiple functional groups, so they are less beneficial for protein predication than amide I region bands (Jackson & Mantsch, 2006).

The changes of secondary structures in mustard proteins under intense thermal processing were shown in Figure 3.5. It was observed that unordered structures were dominant, accounting for 25.72-33.17% of the total protein secondary structure, which will convert to α-helices after thermal processing. The β-sheets were the major secondary structure present in mustard, representing about 19.49- 32.68% of the total secondary structures. 16.9-21.33% of total secondary structures are turns structures, whereas α-helices represents 19.43-30.36%. In comparison to the untreated sample, a noticeable increase in the unordered proteins when treated and decrease in turns was observed when treated from shorter period of time at higher temperature. With increasing time of treatment, higher percentages of β-sheets and α-helices were observed which suggests that the unfolding of turns and aggregation of random coils promotes the formation of β-sheets and α-helices. These findings agree with the results obtained by (Carbonaro et al., 2012). They thermally treated legumes seeds such as common bean, chickpea and lentil. The FTIR analysis showed a reduction in turns and increased β-sheets structures present in lentil. Also, in common beans similar pattern of secondary structure changes were observed. Study by (Zhu et al., 2018) showed the similar results in egg white proteins, In this study, treated egg white proteins was treated with microwave at 60-80 °C for 1-5 min. The FTIR results showed that β-sheet and α-helices structures are sensitive. These all observations are indicative of protein unfolding and/or denaturation. Therefore, depending on the location of allergenic epitopes, time and temperature combination of intense thermal processing of mustard proteins could result in changes in immunoreactivity of treated samples. This may happen due to

unfolding, denaturation and aggregation depending upon whether conformational epitopes are exposed or hidden within molecule structure.

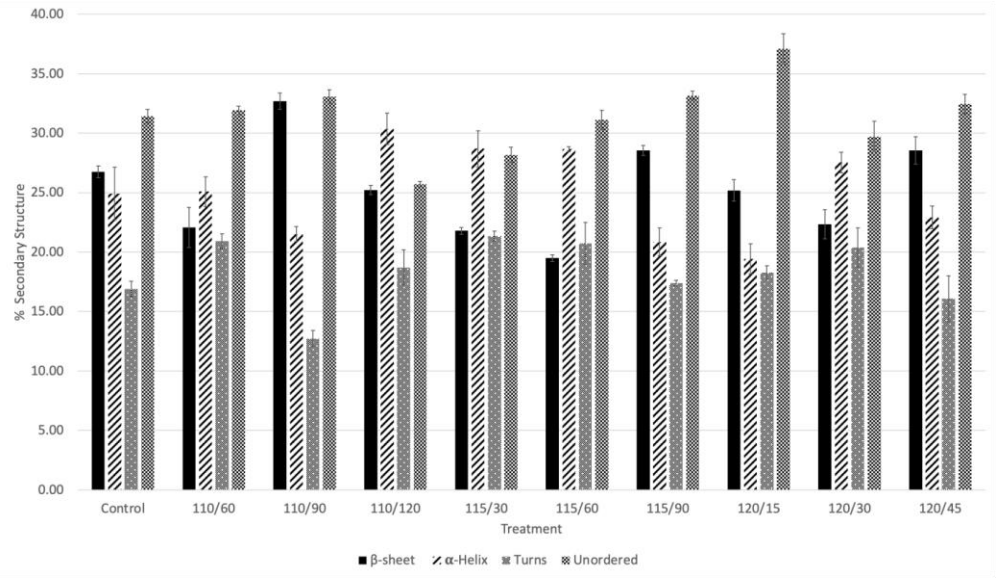

Figure 3.5 Secondary structure in mustard samples under different thermal processing time and temperature conditions

3.4.10. Effect of Enhanced Thermal Processing on Product Quality

3.4.10.1. Color Attributes

Color is an important parameter to consider quality evaluation of mustard products to satisfy the consumer requirements (Newerli-Guz, 2014). Yellow mustard colour is desired for producing a number of food products. The influence of intense thermal processing on the color attributes of mustard extract were shown in Table 3.5. Minor differences in the L*, a* and b* and ΔE values were observed in all the treatments. L value decreased especially when processed at lower temperature. The 120ºC treatment resulted in less changes in L value. Those associated with a and b values were minor. Overall, it was considered that the processing effect on color was not serious, especially in view of the significant advantage one can gain from reduction in allergen immunoreactivity. **ΔE** represents the total color difference which takes in to account the deviations in L, a and b values from the control samples. This represents the changes in an aggregated manner and represents the trend in a majority of cases. It can be seen that the

differences between the control and processed samples were least with samples treated for short duration of time within same temperature conditions and increased with increasing duration of treatment.

Table 3.5 Color and apparent viscosity (at shear rate of 10 s^{-1}) attributes of thermally treated mustard extract samples at different temperature and time combinations

Sample	Temp (°C)	Time (min)	L*	a*	b*	ΔE	Apparent Viscosity (Pa.s)
Control	-	-	14.11 ± 0.04^{a}	01.10 ± 0.21^{a}	06.65 ± 0.19^{a}	NA	$19.2 \times 10^{-3} \pm 0.47^{a}$
Thermal processing	110	60	10.6 ± 0.14^{bcd}	01.78 ± 0.07^{b}	07.57 ± 0.30^{a}	03.97 ± 0.62^{b}	$22.4 \times 10^{-3} \pm 0.33^{b}$
	110	90	9.54 ± 0.25^{cde}	02.37 ± 0.21^{c}	07.48 ± 0.32^{a}	05.03 ± 0.44^{ab}	$28.4 \times 10^{-3} \pm 0.71^{c}$
	110	120	07.54 ± 0.37^{e}	01.69 ± 0.17^{b}	07.74 ± 1.19^{a}	06.85 ± 0.42^{a}	$22.9 \times 10^{-3} \pm 0.97^{b}$
	115	30	12.1 ± 0.37^{ab}	01.81 ± 0.07^{b}	07.78 ± 0.14^{a}	02.74 ± 0.97^{b}	$29.1 \times 10^{-3} \pm 0.06^{cd}$
	115	60	10.4 ± 0.21^{bcd}	00.67 ± 0.28^{a}	06.80 ± 0.30^{a}	04.05 ± 0.33^{b}	$34.1 \times 10^{-3} \pm 0.60^{e}$
	115	90	9.80 ± 0.13^{d}	02.11 ± 0.07^{bc}	06.68 ± 0.10^{a}	04.67 ± 0.19^{ab}	$16.2 \times 10^{-3} \pm 0.47^{f}$
	120	15	12.0 ± 0.70^{abc}	01.73 ± 0.11^{b}	07.70 ± 0.24^{a}	02.80 ± 0.91^{b}	$31.1 \times 10^{-3} \pm 0.13^{d}$
	120	30	11.7 ± 0.54^{bcd}	01.88 ± 0.12^{bc}	07.83 ± 0.17^{a}	03.12 ± 0.89^{b}	$41.4 \times 10^{-3} \pm 0.03^{g}$
	120	45	11.1 ± 0.20^{bcd}	01.93 ± 0.23^{bc}	07.05 ± 0.67^{a}	03.48 ± 0.44^{b}	$25.9 \times 10^{-3} \pm 0.34^{h}$

Data are presented as mean ± SD of three independent observations. The mean difference is significant at the 0.05 level. Different superscript letters within a column indicate significant differences ($p < 0.05$).

3.4.10.2. Rheological Properties

Table 3.5 also shows apparent viscosity values for control and thermally treated samples. The values ranged from 16-40 x 10^{-3} Pa.s between treatments. They were statistically different from each other (Table 3.5) but did not represent a major change from consistency point of view. In centipoise units, this would be in the 20-40 cP range which is higher than water and milk (1-2 cP) but lower than most clear juices like apple juice (50-60 cP), They did not represent any specific pattern either with respect untreated vs different treatments or with respect to temperature or treatment time except for slightly higher values at intermediate treatment times within each temperature.

3.5. Conclusions

Thermal processing at different times and temperatures (110-120ºC, 15-120 min) of mustard slurry had a significant effect on the IR reduction of yellow mustard allergens. ELISA immuno-assay demonstrated that nearly 4 log reductions in mustard allergen concentration was achieved by the thermal processing conditions with Fo in the range 3-10 min. Contrary to this, conventional cooking up to 60 min did not result in a reduction beyond 67%. Protein structure alteration as demonstrated by FTIR results may have led to epitope destruction or masking resulting in changes of IR response. The conditions resulted in only small changes in color and viscosity of the product thereby suggesting a high potential to develop optimized thermal processing conditions which not only reduce the allergen concentrations to ppb levels without significantly impairing the product quality, especially for products in liquid or sauce format.

It should be recognized that allergen reduction may not necessarily mean similar reduction in allergenicity of samples. Allergenicity can only be determined through clinical testing either with animal or human subjects. It is hoped that these identified conditions would provide similar reductions in allergenicity tested through clinical trials. Nevertheless, this study is a clear step forward in processing treatments for reducing the immunoreactivity of allergens, and clearly unique first time study indicating almost four log reduction (>99.9% reduction) in detectable IR in mustard allergens and hoped that this will ultimately benefit the large number of patients who might be allergic to mustard proteins.

Acknowledgement

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PREFACE TO CHAPTER IV

In the previous chapter, effect of enhanced thermal processing on mustard slurry extract allergen immunoresistance activity was evaluated and discussed. This was one side of the spectrum of thermal treatment by increasing the severity of heat in order to gain advantages in immunoreactivity reductions. On the other side, there are various nonthermal treatments which are often used as alternatives to thermal processing. As introduced in Chapter 2, there are several such techniques like high pressure processing, pulsed electric field, pulse light, ozone, plasma and other treatments which give many of the desirable functions of thermal processing without involving excess heat which might degrade the product quality. In this Chapter IV, the research focuses on the biological methods (germination and fermentation) for inducing changes in protein structure and functionality and thereby reducing the immunoreactivity potential of yellow mustard seeds allergens. Five-day germination at 35-40°C and fermentation for 3 days at 25°C and 35°C was performed and ELISA was done to quantify immunoreactivity reduction of seeds. FTIR techniques were used to understand wavenumber shifts and protein's secondary structure of germinated and fermented mustard seeds. Furthermore, these processing methods were combined together and also supplemented with conventional cooking to gain further advantages in reducing in immunoreactivity of mustard allergens. Additionally, the beneficial effect of these treatments on antioxidants and total phenols were also evaluated.

All the experimental work and data analysis were conducted by the candidate under the supervision of Dr. H. S. Ramaswamy.

Part of this study has been used for presentations and publications as follows:

Jawanda S.K., Sarhangpour R., H.S. Ramaswamy. 2021b. Tracking immunoreactivity of yellow mustard allergens through seed germination and lactic acid fermentation. Prepared for submission to Journal of Food Science and Technology. *(Paper in review)*

Jawanda S.K., Ramaswamy H.S. (2021). Impact of selected biological methods on fate of allergens in yellow mustard seeds. Northeast Agricultural and Biological Engineering Conference (NABEC), July 2021, Virtual on Zoom. (Poster Presentation)

CHAPTER IV
TRACKING IMMUNOREACTIVITY OF YELLOW MUSTARD ALLERGENS THROUGH SEED GERMINATION AND LACTIC ACID FERMENTATION

4.1. Abstract

Food allergens are becoming increasingly menacing and disrupting the health and social structure of a significantly large population worldwide. Mustards are among the well recognized food allergens which affects many sensitive individuals. Many food processing methods are continually being explored to reduce allergen immunoreactivity and for developing hypoallergenic functional foods, among them thermal processing being the most common one. Other than cooking, generally, as a nonthermal alternative, it is recognized that biological processing methods such as germination and fermentation may help to attenuate the immunoreactivity of food allergens especially involving seeds like plant foods like mustard. The objective of this study was to evaluate the effect of seed germination and lactic acid fermentation on allergens of yellow mustard seed immunoreactivity (IR) using commercial sandwich ELISA assay kits. Samples from five-day germination at 35-40°C and three-day fermentation at 25°C and 35°C were evaluated. The germination and fermentation processes resulted in varying reductions in immunoreactivity with their combinations yielding 90% reduction that is one log reduction in total. When complemented with further stove top cooking, further reduction by almost 98% was possible while cooking alone only resulted in about 70% reduction as well as log reduction in immunoreactivity increased from 0.23 to 1.74. FTIR results confirmed that changes in mustard protein conformation maybe due to unfolding and/or denaturation of mustard proteins. These processing methods were beneficial as they not only helped reduce the allergen immunoreactivity, but also increased the antioxidant activities in germinated and fermented mustard seeds.

4.2. Introduction

Mustard is used in preparation of various food products such as processed meats, salad dressings, seasoning blends, sauces, condiments, and pickled products in order to enhance flavor and for its nutritional values (Lee et al., 2008). Mustard seeds are the main ingredient of north-American-style mustards. Mustard seeds are also major ingredients in Chinese and European-

style mustards (AAFC, 2009a). Mustard is recently added to the list of priority allergens in European Union as well as Canada (World Health Organization, 2010). Mustard allergy cases are reported to cause allergic symptoms and reactions such as oral allergy syndrome, immediate skin response and more severe reactions such as anaphylactic shock in hyper-sensitive persons (Caballero et al., 2002). There is no successful treatment for allergy remediation and avoiding the products containing mustard is the only real solution to avoid allergic reaction in sensitive individuals. Known yellow mustard allergens associated with allergic reactions are Sin a 1, Sin a 2, Sin a 3 and Sin a 4. Out of these, Sin a 1 and Sin a 2 are major allergens characterized as seed storage proteins belonging to 2S albumin and 11S globulin family, respectively (Menendez-Arias et al., 1988l Palomares et al., 2007) whereas Sin a 3 and Sin a 4 are a non-specific lipid transfer protein (nsLTP) and a profilin, respectively (Sirvent et al., 2009).

Food processing results in matrix interaction, structural changes and/or solubility changes, and may have effect on allergenicity of final product (Khuda et al., 2015). Processing can affect allergic proteins and result in decrease, increase or no change on immunoglobulin E (IgE) binding capacity of allergen (Verhoeckx et al., 2015). Food processing methods including thermal and non-thermal treatments and type of treatment may differ in its effect on epitopes. Thermal processing may be achieved by dry heat (for e.g., roasting, frying, infrared heating) or using wet heat (for e.g., autoclaving, boiling blanching, pressure cooking, ohmic heating, MW/RF heating, extrusion). Non-thermal processing includes high pressure processing, grinding, milling, and dehulling and dehusking to name a few (Sathe et al., 2005). Processing may result in alteration that permits unmasking or making of allergenic epitopes which may reduce, enhance, or have no effect on allergenicity of the offending food. Some treatments like biological, biochemical, or chemical applications, may influence the degree of immunoreactivity. These methods include treating with additives, brining, curing, enzymatic treatment, fermentation, germination, pickling and salting (Sathe et al., 2016). However, there has been no effective method to eliminate allergenic capacity of mustard. In Chapter 3, one such technique involving enhanced thermal treatment was successfully developed and discussed. Developing new minimal processing approaches to modify allergen content will have a significant additional advantage.

Recently, several studies had demonstrated that during germination, the seed storage proteins, including allergenic proteins, breakdown into peptides or amino acids by catalytic

enzymes that are responsible for providing nitrogen required for seedling growth (Kang et al., 2007; Wu et al., 2012). Depending on specificity of enzymes and epitopes susceptibility to the active enzymes, germination may result in elimination of certain epitopes in seed storage proteins during germination period (Daussant et al., 1976). There are reported findings on rice and soybeans showing significant degradation of storage protein and reduction of immunoreactivity after short-term germination (Wu et al., 2012). Traditionally, legume sprouts are very popular in eastern countries and are getting increasingly acceptable in western countries consumers. There are a number of studies demonstrating that sprouts are novel functional foods and can act as dietary source of phenolic substances (Wang et al., 2017). With short-term germination, the antioxidant polyphenols increase in legume seeds (Li et al., 2014).

Furthermore, scientific information on effects of fermentation on food allergens is very limited. Fermentation is considered a method to decrease immunoreactivity as demonstrated by studies done on fermenting soybean, skim milk and whey proteins (Chen et al., 2012). Lactic acid fermentation can decrease immunoreactivity of soya and it has potential of developing nutritious hypoallergenic soya products (Frias et al., 2008) (Song et al., 2008). Combined strains of *Lactobacillus helveticus* and *Streptococcus thermophilus* were the most effective in reducing the antigenicity of whey proteins (Bu et al., 2010). However, fermentation is a natural process which not only results in lowering the immunoreactivity but also improves antioxidants and total phenols content of food product (Shekib, 1994). The application of one single processing technique may not show a significant reduction in immunoreactivity of allergic foods. The combination between various processing techniques can provide a new strategy for decreasing immunoreactivity (Dong et al., 2021). Research studies focusing on allergy reduction of mustard proteins using nonthermal methods are very scarce or at very early stages.

Enzyme-linked immunosorbent assay (ELISA) is used for allergen detection and quantification as it is very sensitive, simple, rapid and accurate method (van Hengel, 2007). Sandwich ELISA is employed for identification and quantification of allergic proteins (Shim & Wanasundara, 2008). Additionally, changes in protein structure are studied by Fourier transform infrared spectroscopy (FTIR). Amide I region of infrared spectrum are evaluated for protein secondary structure changes and used to understand deviations of secondary structure such as conformational changes that is unfolding and/or folding of protein and formation of aggregates (Carbonaro et al., 2012). Presently, there is very limited work published on demonstrating the

effect of food processing technologies on yellow mustard proteins. Published information on qualitative changes is abundant on denaturation and alteration of functional properties but studies on quantifying the allergens in yellow mustard is almost nonexistent. Moreover, relation between IR and structural properties of mustard proteins has been not explored in detail. Fourier transform infrared spectroscopy (FTIR) is a useful technique to provide information on germination and fermentation effects on the secondary structure of mustard proteins which can be related to the IR changes of mustard proteins.

Therefore, the aim of this study was to (1) track the reduction in immunoreactivity of mustard proteins through the nonthermal process approach of seed germination and lactic acid fermentation of yellow mustard seeds (independently, and in combination, with and without added stove top finish cooking) using sandwich ELISA technique (2) to use FTIR spectroscopy to understand conformational changes induced by the biological methods (germination and fermentation) and its correlation with the observed immunoreactivity values and finally (3) investigate the effect of these processing treatments on the antioxidant capacities of yellow mustard seeds.

4.3. Material and Methods

4.3.1. Mustard Samples

Yellow mustard seeds (*Sinapis alba*) were purchased from Food to Live Company (Brooklyn, NY, USA).

4.3.2. Seed Germination

The experimental samples were prepared according to the method adapted from (Michalcová et al., 2021). 25 g yellow mustard seeds were soaked in 50 mL of water for 24 h at 25°C. Then, the seeds were weighed, tossed, and spread on plastic trays. Mustard seeds on each tray were incubated within the germinator (Kikiheim automatic bean sprouts machine, 25.5 × 34 cm and try aperture-1mm with 2-layer germination tray) in dark at temperature in the range of 35°C to 40 °C controlled by germinator's operational settings and 90% relative humidity for 0 to 5 day(s) of germination. All the samples were ground and freeze-dried at -50ºC in a freeze drier (Labconco Corporation, Kansas City, MO) for further analysis.

4.3.3. Lactic Acid Fermentation

The preparation of experimental sample was carried out by the solid-state fermentation of mustard seeds (Song et al., 2008). Raw mustard seeds were suspended in sterile distilled water (1:1, w/v) and kosher salt was added at a ratio of 1:25 (w/w). Further, a mixed culture of active LAB strains i.e. L. *plantarum*, Ln. *mesenteroides* and Pc. *acidilactici* (2.4×10^8 CFU/g) starter culture (Starter kit from Cutting Edge Cultures LLC, Wakefield, RI) and inoculated at 0.1% (w/w) in raw seeds. This kind of fermentation is used to prepare European-style mustards and traditional fermented food in Taiwan (Chen, Yanagida, & Hsu, 2006) (World Health Organization, 2010). The fermenting samples were incubated at 25°C and 35°C and a relative humidity of 90% and fermented for 1 to 3 day(s). All the samples were then ground in a pestle mortar and freeze-dried at -50ºC in a freeze drier (Labconco corporation, Kansas City, MO) for further analysis.

4.3.4. Sandwich ELISA

A sandwich ELISA kit (3M Mustard Protein ELISA Kit, Maplewood, Minnesota, USA) was used for detection and quantitative analysis of mustard protein allergens. Sandwich ELISA uses of two matching antibody pairs (detection and capture antibodies). This ELISA kit contained a microtiter plate coated with an anti-mustard antibody, and reagents including mustard protein standard concentrate, mustard horseradish peroxidase (HRP) conjugate, an extraction buffer, diluent solution, wash solution, chromogenic substrate solution, and stop solution. Mustard proteins present in the test samples are extracted by the extraction buffer and it reacts with anti-mustard antibody that is adsorbed onto surface of polystyrene microtiter wells. Removal of unbounded fractions is done by washing with wash solution and anti-mustard antibodies conjugated with horseradish peroxidase (HRP) prepared with diluent solution are added. These enzymes, labelled antibodies, form complexes with previously bound mustard proteins. This is followed by second washing step, and then the enzyme bound to immunosorbent is detected by adding a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB) and stop solution is added to stop reactions. Color is developed by the enzymatic reaction, and it varies directly with the concentration of absorbed mustard protein in sample tested. This procedure has been detailed in Lee et al. (2008). Then final absorbance of mustard protein in each test sample is recorded at 450 nm as a measure of the concentration of mustard protein in the test sample. The

concentration of mustard protein is calculated according to the standard curve obtained by the spiking different concentrations of supplied standards in the kit in the same manner and reading the absorbance (Okolie et al., 2018).

4.3.5. Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) was used to study the changes in secondary structure present in mustard protein. The freeze-dried sample (0.1 g) was transferred to the diamond crystal at room temperature. The data was analyzed by Windows-based OMNIC software (Version 9, thermo Nicolet Co., Madison, WI) using Nicolet™ iS50 FTIR Spectrometer with ATR accessory (Nicolet Co., Madison, WI) equipped with a computer. A total of 128 scans of FTIR spectra at a resolution of 4 cm^{-1} were recorded and averaged in the mid-infrared region (4000-500 cm^{-1}). To avoid the influence of air, a background spectrum without the sample was also collected before each determination. The spectra were deconvoluted and were used for calculating percentage of the secondary structure in Amide I region of mustard protein with a bandwidth of 31 cm^{-1} and an enhancement factor of 2.4 (Achouri & Boye, 2013).

4.3.6. Conventional Cooking of Treated Samples

In order to compare with conventional cooking, selected germinated and fermented samples were freeze dried and the freeze dried flours were used to prepare a 5% slurry by mixing selected freeze-dried samples with double distilled water. These samples were also cooked in boiling water at 100ºC for 30 and 60 min to evaluate the effect of cooking under atmospheric processing conditions. The slurry sample was filtered through Whatman #4 filter paper and the clear extract was analysed.

4.3.7. Phenolic Compounds and Antioxidant Activity

4.3.7.1. Extraction of Phenolic Compounds

The approach to extract phenolic compounds was adapted from the method outlined by (Marathe et al., 2011). 0.1 g of raw and freeze-dried germinated and/or fermented samples were dissolved in 10 ml of 100% methanol (Millipore Sigma Canada Ltd, Oakville ON). Then, these samples were placed in shaking water bath (Julabo USA, Inc., 884 Marcon Boulevard, Allentown, PA) at 100 rpm and incubated for 2.5 h at room temperature (28.2°C). Afterwards,

the mixture was centrifuged at 3000×g for 15 min. The supernatant was filtered and used for antioxidant scavenging activity and total phenolic content assay.

4.3.7.2. DPPH Antioxidant Scavenging Activity

The free radical scavenging activity of all samples was determined by DPPH (2,2-diphenyl-1-picrylhydrazyl) method described by (Peters et al., 2011). A fresh solution of DPPH (0.1 mM) in methanol was first prepared. 0.2 ml of methanolic extract of each sample was added to 3 ml DPPH methanol solution. The sample solutions were vortexed and left to incubate at room temperature for 30 min in dark. Thereafter, the absorbance value was measured at 517 nm by using UV/VIS spectrophotometer (VWR, Model V-3100PC). The control for this assay was prepared by adding 0.2 ml methanol to 3 ml of DPPH. The assay was performed in triplicates. The percentage scavenging activity was calculated using the following equation:

$$\% \text{ Scavenging activity} = \frac{Abs\ (Control) - Abs\ (Sample)}{Abs\ (Control)} \times 100$$

where:

Abs (Control) is the absorbance of DPPH solution in methanol;

Abs (Sample) is the absorbance of DPPH solution mixed with sample.

4.3.7.3. Total Phenol Content

The determination of total phenol content was based on Folin-Ciocalteu method (Madaan et al., 2011). Initially, 0.5 ml of methanol was added to 0.5 ml of methanolic sample extract. Subsequently, 5 ml of Folin-Ciocalteu reagent (diluted 10 times with double distilled water) and 5 ml of aqueous sodium carbonate solution (7.5%, w/v) was added to the reaction mixture. Afterwards, the sample solutions were vortexed and incubated for 20 minutes at room temperature. The absorbance value was measured at 760 nm against blank (prepared in same way but replacing the sample with methanol). The standard curve range was 50-350 μg/ml gallic acid (R^2 = 0.9988). The results were expressed as mg of gallic acid equivalent (GAE)/g dry weight (DW).

4.3.8. Statistical Analysis

The data were analyzed by one-way analysis of variance (ANOVA), followed by Post-hoc using Tukey's test ($p < 0.05$) using an SPSS 27.0 analytical software (SPSS Inc., Chicago, USA). All treatments and experimental studies were conducted in triplicates.

4.4. Results and Discussion

4.4.1. Germination Studies

4.4.1.1. Immunoreactivity Reduction

The immunoreactivity of allergic mustard protein was quantified by forming a standard curve by spiking mustard allergen standard provided in the sandwich ELISA kit. Dilution of samples were done to bring the concentration within the range established by the nonlinear standard curve fitting a second order polynomial (quadratic model). Allergen concentration was calculated from standard curve in ppb (ng/L) and multiplied with dilutions factors used (Abbott et al., 2010).

This assay was performed to assess the effect of seed germination. The results presented in Table 4.1 showed the declining IgE binding capacity of germinated mustard seeds. The results suggest that IgE binding capability start to decline after 24 h of soaking. Additionally, these results confirmed that allergic protein content in mustard sprouts decreased rapidly after day 1 of germination (84%), but gradual further reduction in immunoreactivity in next days of germination (87%) that is 0.9 log reduction in immunoreactivity. The five-day germination of yellow mustard seeds confirms that it has positive effect on IgE binding capacity of mustard. In the previous study findings of assessing the effect of germination on IgE binding capability of peanut, it was observed that IgE binding begin to decrease after soaking. It exhibited a downward trend during first four days of germination which was significant than raw or soaked peanut samples (Rao et al., 2018). These results also demonstrated that approximately 87% total reduction of immunoreactivity of mustard seeds was there because of five-day germination process as shown in Figure 4.1. Similarly, results on soyabean germination reported large reduction at 72 h after seed germination (Wu et al., 2012).

Table 4.1 Mustard allergen concentration of germinated and fermented mustard allergens

Type of Treatment	Temperature (°C)	Time Day(s)	Mustard Allergen Concentration (ppm)	Reduction in IR [log(10)]
Control	-	-	$246 \times 10^3 \pm 1.42^{a}$	-
Germination (G)	35-40	0	$232 \times 10^3 \pm 1.81^{b}$	0.03
		1	$38.7 \times 10^3 \pm 0.54^{c}$	0.80
		2	$36.3 \times 10^3 \pm 1.31^{cde}$	0.83
		3	$36.1 \times 10^3 \pm 0.25^{cde}$	0.83
		4	$34.1 \times 10^3 \pm 0.46^{cef}$	0.86
		5	$31.3 \times 10^3 \pm 0.33^{ef}$	0.90
Fermentation (F)	35	1	$36.4 \times 10^3 \pm 0.37^{cde}$	0.83
		2	$35.4 \times 10^3 \pm 0.55^{de}$	0.84
		3	$33.9 \times 10^3 \pm 0.32^{ef}$	0.86
	25	1	$37.5 \times 10^3 \pm 0.25^{cd}$	0.82
		2	$36.7 \times 10^3 \pm 0.27^{cde}$	0.83
		3	$35.1 \times 10^3 \pm 0.22^{de}$	0.85
G & F	G (35-40), F (35)	G-5, F-3	$24.4 \times 10^3 \pm 0.21^{g}$	1.01

Data are presented as mean ± SD of three independent observations. The mean difference is significant at the 0.05 level. Different superscript letters within a column indicate significant differences ($p < 0.05$).

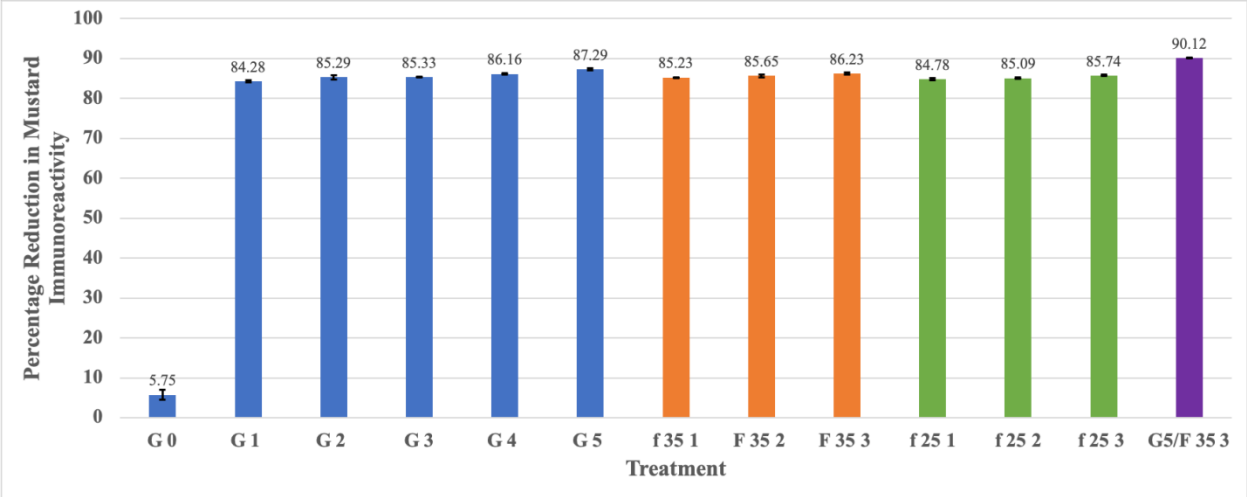
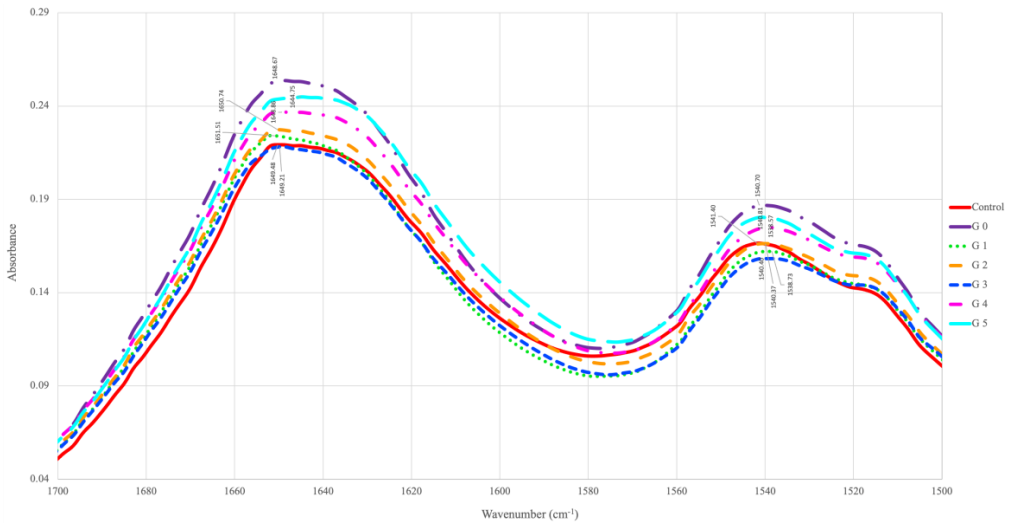


Figure 4.1 Percentage reduction in mustard immunoreactivity of germinated and fermented mustard seeds

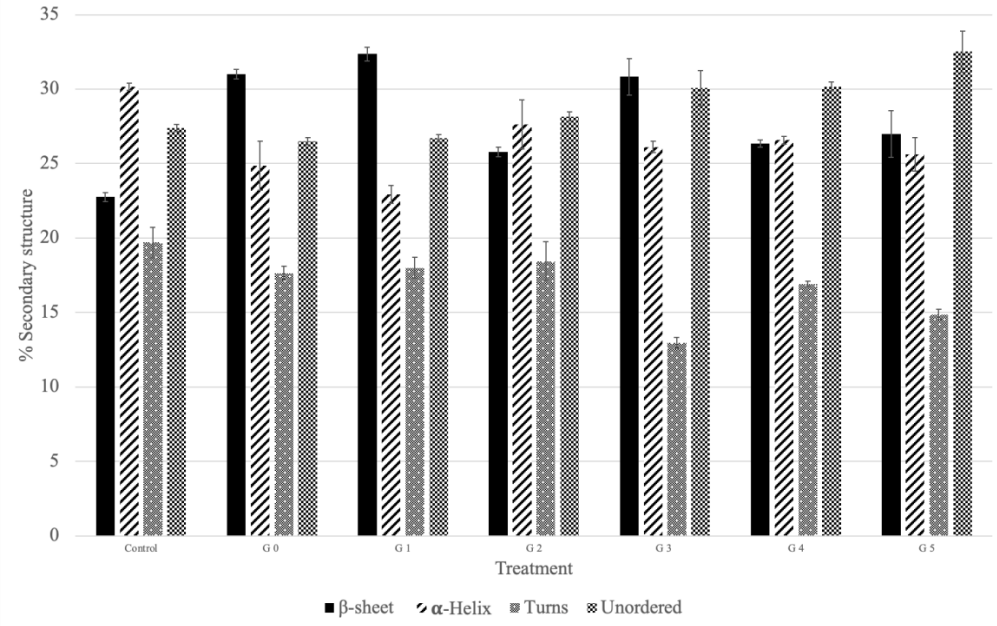
4.4.1.2. Fourier Transform Infrared Spectroscopy (FTIR)

Fourier-transform infrared spectrometry (FTIR) analysis is certainly most important analytical techniques to study conformational changes in amide I and amide II region of processed samples. The amide I and amide II region are most important for interpreting vibrational bands of protein backbone (Huson et al., 2011). To study the protein secondary structure changes that are conformational changes (folding and/or unfolding of protein and formation of aggregates), amide I region deviations of the infrared spectrum are used (Carbonaro et al., 2012). This region is associated with C=O stretching of peptide backbone and N-H bending vibrations (Ahmed et al., 2017). Amide I comprise of overlapping bands of number of secondary structures that are β-sheets, α-helices, turns, and randomly coiled conformations (Wang et al., 2018). Deconvolution of amide I bands isolated each band and differentiated its frequency to determine right secondary structure components and quantify it (Long et al., 2015) (Martínez-Velasco et al., 2018). The amide I region bands are assigned as β- sheets (1613-1637; 1682-1696), α- helices (1645-1662), turns (1662-1682, 1630), and unordered (1637-1645) (Dong et al., 2020). The typical FTIR spectra of amide I and amide II of raw and germinated mustard seeds are shown in Figure 4.2(a).

The FTIR spectra band observed at 1700-1500 cm^{-1} for raw and germinated mustard seed samples corresponded to amide I and amide II group of proteins. The vibrations observed for mustard samples showed slight variation in wavenumber in germinated mustard seeds samples as compared to raw mustard sample. This could be due to unfolding and/or denaturation of proteins and modification of protein chain during germination process. Similar observations were also reported by (Sofi et al., 2020), the wavenumber shifts were observed in amide region for germinated chickpea flour when compared to native flour. These slight differences observed in protein region (1700-1600 and 1545-1535 cm^{-1}) corresponds to protein degradation during processing treatments (Kumar et al., 2021). The changes occurring in secondary structure of mustard proteins when undergone germination process were shown in Figure 4.2(b). It was observed that percentage of β-sheets and unordered structures increased with five-day germination of raw mustard seeds. Whereas reduction in α-helices and turns percentages were recorded. These findings suggest that germination process is responsible for the secondary structure changes in amide I region. Similar results were observed by (Kaur & Gill, 2021) in case of germinating cereal grains.



a



b

Figure 4.2 FTIR-spectra of Amide I and II components (a) and secondary structure (b) of raw and germinated mustard seeds

4.4.2. Fermentation Studies

4.4.2.1. Immunoreactivity Reduction

Table 4.1 shows that allergic mustard protein content in fermented mustard seeds deceased rapidly after first day of fermentation, but declined slowly in next stages when fermented both at 25°C and 35°C. It was observed that with 3 days of mustard seed fermentation

decreased immunoreactivity approximately more than three-forth as compared to that at 0 day. The concentration of mustard protein was lower when mustard seeds were fermented at 35°C than fermenting at 25°C. In the control samples with mustard allergen concentration value of 246 $\times 10^3$ ppm, value reduced to 33.9 $\times 10^3$ when fermented at 35ºC for 3 days and 35.1 $\times 10^3$ when fermented at 25ºC for 3 days that is 0.86 and 0.85 log reduction respectively. The fermentation results suggest that significant reduction ($p < 0.05$) in immunoreactivity and shows efficacy of mixed culture to reduce immunoreactivity resulting in more than 84% reduction demonstrated in Figure 4.1. According to (Frias et al., 2008), Fermented soyabean have significantly reduced immunoreactivity with induced fermentation. This study also suggested that L. *plantarum* culture exhibits a better potential for development of reduced immunoreactive fermented soyabean as compared to mold strains. In another study, both natural and induced fermentation results showed reduced IgE binding capacity of nearly 89% in soyabean meal (Song et al., 2008).

In a previous study by (Kleber et al., 2006) also suggest LAB potential to reduce antigenic response to reduce antigenic response in whey and skim milk. Additional synergic effect of immunoreaction reduction was demonstrated when a mixture of LAB and *S. thermophilus* strains were used. Combined strains effect on whey proteins during fermentation was also observed by (Bu et al., 2010) and also demonstrated that fermentation by lactic acid bacteria as effective way to decrease antigenicity of whey proteins.

4.4.2.2. FTIR Results

In the case of fermentation, minor shifts in wavenumbers (cm^{-1}) were observed in both fermenting mustard seeds at 35°C and 25°C as shown in Figures 4.3(a) & 4.3(b) respectively. Wavenumber variation in fermentation duration (day 1-3) was more prominent when fermented at 35°C. Whereas, fermenting at 25°C resulted in less evident variation of wavenumbers from raw mustard samples. Therefore, fermenting mustard seeds at 35°C using mixed strains culture was more beneficial than fermenting at 25°C and resulted in more C=O stretching vibrations in amide I region. Similar changes in amide region were observed by (Sadh et al., 2018) and results also revealed the significant increase in protein content during fungal fermentation by A. *oryzae*. In a pervious study, it was observed that solid state fermentation with A. *niger* resulted in increase of C = O stretching of amide groups in proteins of rapeseed meal (Shi et al., 2016). In

case of fermented broccoli samples, the peaks at 1550 and 1640 cm^{-1} were associated with amide-stretching bands of proteins (Ye et al., 2019).

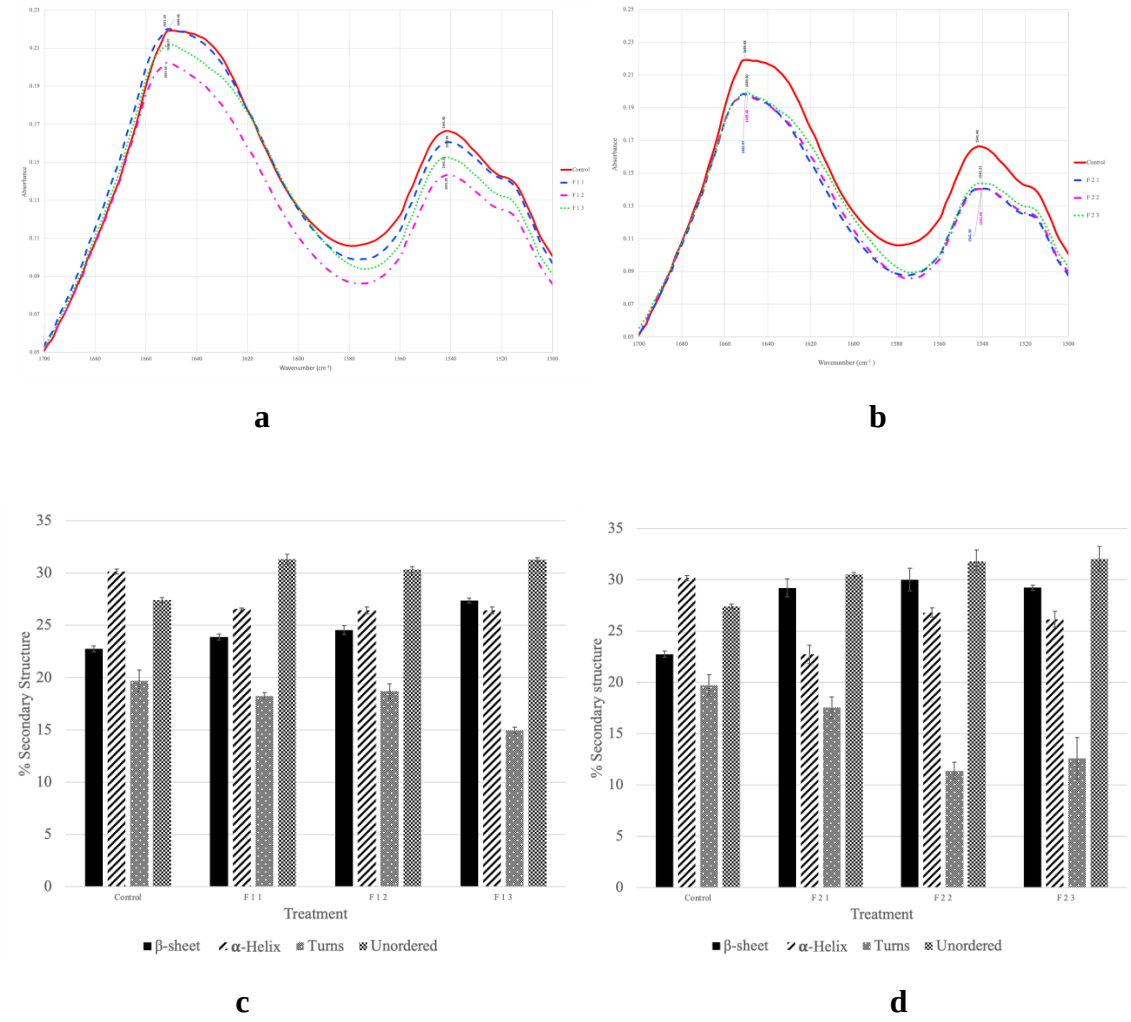


Figure. 4.3 FTIR-spectra of Amide I and Amide II components of raw and fermented (35°C) mustard seeds (a), raw and fermented (25°C) mustard seeds (b), Secondary structure in raw and fermented (35°C) mustard seeds (c), raw and fermented (25°C) mustard seeds (d).

The fermentation process also resulted in similar secondary structure changes as observed in case of germinating mustard seeds and demonstrated in Figures 4.3(c) & 4.3(d). Three-day fermentation resulted in increase in β-sheets and unordered structures and decreased percentages of α-helices and turns both in case of 25°C and 35°C. These results agree with study done by (Luan et al., 2021) which showed the level of β-sheet structure increased in secondary structure

of fermented horseradish sauce, while α-helices percentage decreased when compared with unfermented samples.

4.4.3. Combination of Germination and Fermentation Treatments

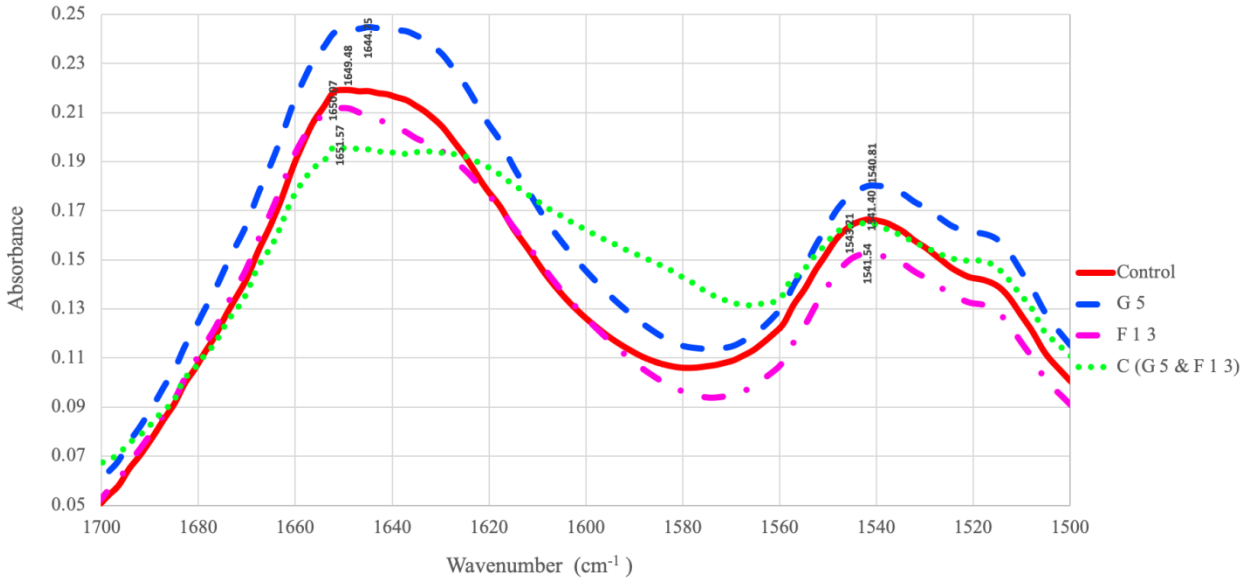
4.4.3.1. Immunoreactivity Reduction

A remarkable reduction in detectable mustard protein was observed when best combination of both processing techniques were combined resulting final value of mustard allergen concentration as 24.4×10^3 ppm decreased from 246×10^3 ppm that means one log reduction in immunoreactivity as shown in Table 4.1. This means it resulted in more than 90% reduction in immunoreactivity of mustard as it is evident in Figure 4.1. The combined effect of both methods showed additional benefit in immunoreactivity reduction than when applied separately. Therefore, the biological methods seem to be very effective and demonstrated the potential to explore such processing methods to reduction the allergen immunoreactivity.

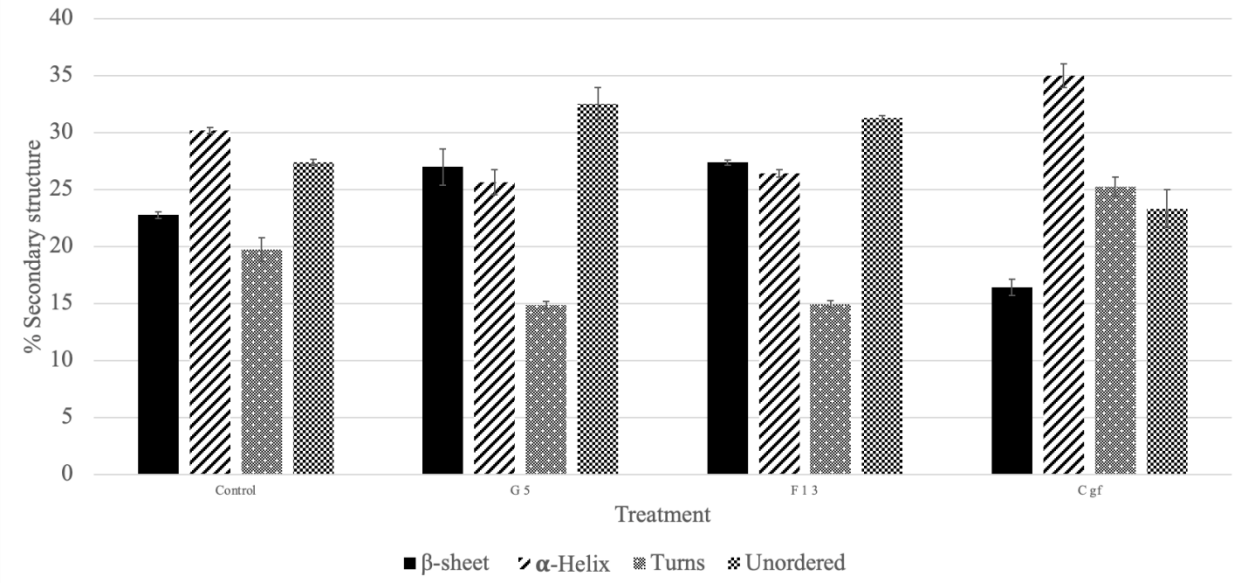
4.4.3.2. FTIR Results

Figure 4.4(a) represents FTIR spectra for combined treatment of 5-day germination followed by fermentation at 35°C for 3 days and raw mustard seed samples. Major variations in amide I and amide II regions are observed when both germination and fermentation methods are applied to yellow mustard seeds.

The secondary structure changes obtained from FTIR results for mustard seeds samples treated with both processes that is germination and fermentation are shown in Figure 4.4(b). Comparing the combined treatment samples that germination for 5 days followed by fermentation (35°C) for 3 days resulted in largest increase in percentages of α-helices and turns and decrease in β-sheet and unordered structures. Therefore, the FTIR results showed that secondary structure of mustard proteins are sensitive to biological processing methods. These treatments are promising methods for bringing changes in secondary structure of mustard proteins.



a



b

Figure 4.4 FTIR-spectra of Amide I and Amide II components (a) and secondary structure (b) in raw and germinated (5 days), fermented (35°C) mustard seeds

4.4.4. Conventional Cooking

The conventional cooking of prepared 5% extract slurry of selected freeze-dried mustard samples showed reduction up to 0.48 ppm as compared to control sample value of 26.6 ppm as

described in Table 4.2. In case of 5-day germinated mustard samples, immunoreactivity reduced from 26.6 ppm to ~1 ppm, after cooking for 60 min. Samples prepared from mustard seeds fermented for 3 days at 35ºC also showed final immunoreactivity of 1.12 ppm after 60 min cooking. The highest reduction was observed for cooked samples prepared from the best combination of both biological methods with the end value of mustard allergen concentration as 0.48 ppm after cooking for 60 min

Considering the percentage reduction as shown in Figure 4.5, the control sample treated for 30 min and 60 min resulted in approximately 41% and 68% reduction in immunoreactivity respectively. This percentage reduction corresponds to 0.23 and 0.50 log reduction in immunoreactivity when cooked for 30 min and 60 min respectively. For germinated mustard samples, percentage reduction in immunoreactivity of cooked slurry samples ranged 91-96% approximately. Additionally, in this case highest log reduction is observed for cooked slurry extract prepared from germinated day 5 sample that is 1.40 log reduction in immunoreactivity. On the other hand, fermentation showed similar reduction with 3-day fermentation at 35ºC showing final immunoreactivity reduction at 95.8% which translates to 1.37 log reduction. Also, samples prepared from best combination of both biological methods was applied to thermal processing showed highest reduction in mustard allergen immunoreactivity resulting 96.7% reduction on 30 min heating and 98.2% reduction when heated for 60 min as well as reaching to log reduction of 1.48 and 1.74 in immunoreactivity respectively.

Thermal treatment can cause modifications in allergic protein structure due to denaturation, peptide bond hydrolysis and aggregation by covalent and disulfide bonds and extent of effect depends upon duration and severity of processing (Maleki & Sathe, 2006). Since these conventional cooking treatments can induce further changes in binding of allergic mustard proteins that may resulted in lowering immunoreactivity intensities in treated samples.

4.4.5. Antioxidant and Total Phenolic Content

4.4.5.1. DPPH Antioxidant Scavenging Activity

Considering the antioxidant activity, significant differences were observed in the percentage radical scavenging activity depending on the processing method applied to the yellow mustard seeds. It was observed the sprouting resulted in an improved percentage radical scavenging activity increasing from 24.2% to 32.1% after 5 days of germination. This implies

that ability of sprouted mustard seeds to scavenge free radicals increases after the germination process. The antioxidant properties of germinated black *(Brassica nigra)* and brown *(Brassica juncea)* mustard seeds were improved in the study by (Borș et al., 2017). In case of fermenting mustard seeds, a higher DPPH antioxidant scavenging activity was observed as compared to their seeds counterparts as shown in Table 4.3.

Table 4.2 Mustard allergen concentration of conventionally cooked 5% slurry prepared from freeze dried germinated and fermented mustard allergens

Type of Treatment	Temperature (°C)	Time (min)	Mustard Allergen Concentration (ppm)	Reduction in IR [log(10)]
Control	-	-	26.6 ± 0.59^{a}	-
Control	100	30	15.6 ± 0.2^{b}	0.23
	100	60	08.5 ± 0.6^{c}	0.50
Germination (day 4)	100	30	2.20 ± 0.0^{d}	1.08
	100	60	1.93 ± 0.27^{d}	1.14
Germination (day 5)	100	30	1.53 ± 0.18^{ef}	1.24
	100	60	1.07 ± 0.11^{fg}	1.40
Fermentation (35ºC) (day 2)	100	30	1.77 ± 0.06^{de}	1.18
	100	60	1.23 ± 0.04^{efg}	1.33
Fermentation (35ºC) (day 3)	100	30	1.57 ± 0.22^{ef}	1.23
	100	60	1.12 ± 0.34^{fg}	1.37
Germination (day 5) & Fermentation (35ºC)(day 3)	100	30	0.89 ± 0.05^{gh}	1.48
	100	60	0.48 ± 0.12^{h}	1.74

Data are presented as mean ± SD of three independent observations. The mean difference is significant at the 0.05 level. Different superscript letters within a column indicate significant differences ($p < 0.05$).

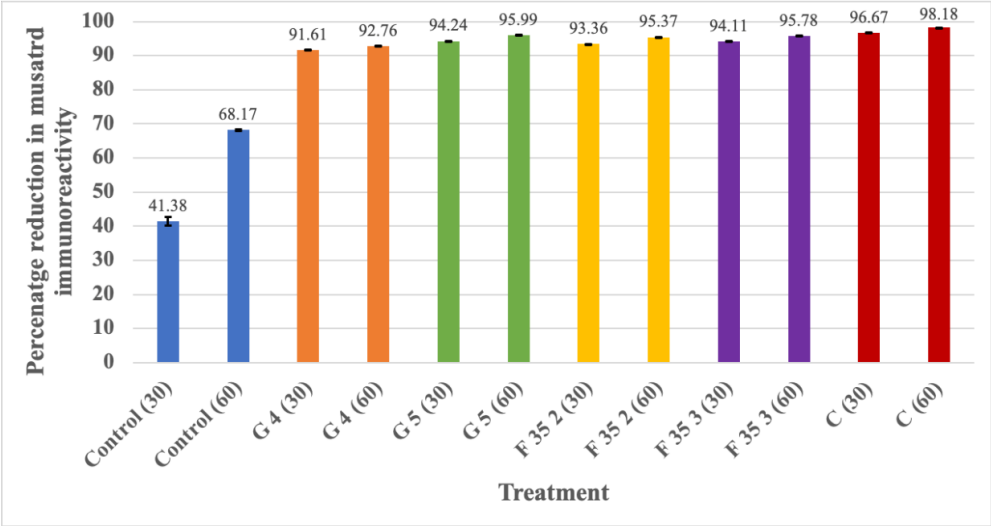


Figure 4.5 Percentage reduction in mustard immunoreactivity of cooked 5% extract slurry of selected germinated and fermented mustard seeds

Comparing fermentation at 25ºC and 35 ºC to control higher ability to scavenge DPPH free radicals was elicited at 35ºC. Results on antioxidant activities in previous reports by (Wang et al., 2006) and (Rekha & Vijayalakshmi, 2008) showed an increasing trend in case of soymilk fermentation using pure or mixed lactobacillus cultures. Therefore, these biological methods demonstrated enhanced capability of free radicals scavenging activity and further combined effect of both methods resulted in highest % scavenging of DPPH free radicals i.e. 33.2%.

4.4.5.2. Total Phenolic Content

Total phenol contents were assessed for germinated and fermented mustard samples are described in table 4.3. It was observed that the germination process increased the phenolic compounds in mustard sprouts. An increase (from initial 3.49 to final 7.92 mg GAE/g) was reported after 5 days of germination at 35-40°C. (Borș et al., 2017) observed that the change in phenol content is a function of the germination duration. Similar to germination, an increasing trend in total phenols is observed throughout fermentation process. Since, higher total phenols were observed in case of fermentation at 35°C than fermenting at 25°C, it was confirmed that increase in phenolic content is a function of fermentation temperature. According to (Huang et al., 2012), pickled and dried mustard extract showed a high amount of total phenols and a good

sources of antioxidants. As germination process increased the total phenols followed by fermentation process, which resulted in further rise in phenolic content reaching their maximum i.e., 8.16 mg GAE/g.

Table 4.3 presents percentage radical scavenging activity and total phenolic content of methanolic extract of prepared mustard samples

Type of Treatment	Temp (°C)	Time Day(s)	% Radical Scavenging Activity	Total phenolic content (mg GAE/g sample)
Control	-	-	24.20 ± 0.24^{a}	3.49 ± 0.01^{a}
Germination (G)	35-40	0	28.59 ± 0.22^{bc}	3.85 ± 0.02^{b}
		1	29.16 ± 0.11^{bcd}	5.85 ± 0.07^{f}
		2	30.46 ± 0.08^{def}	6.72 ± 0.05^{g}
		3	31.48 ± 0.06^{ef}	7.09 ± 0.02^{h}
		4	31.89 ± 0.26^{fg}	7.76 ± 0.03^{i}
		5	32.05 ± 0.09^{fg}	7.92 ± 0.02^{i}
Fermentation (F)	35	1	28.62 ± 0.13^{bc}	5.50 ± 0.01^{e}
		2	29.89 ± 0.13^{cde}	6.98 ± 0.04^{h}
		3	30.69 ± 0.09^{def}	7.04 ± 0.15^{h}
	25	1	24.69 ± 0.03^{a}	4.57 ± 0.10^{c}
		2	25.68 ± 0.04^{a}	5.08 ± 0.02^{d}
		3	28.07 ± 1.96^{b}	5.49 ± 0.06^{e}
G & F	G (35-40), F (35)	G-5, F-3	33.15 ± 0.06^{g}	8.16 ± 0.02^{j}

Data are presented as mean ± SD of three independent observations. The mean difference is significant at the 0.05 level. Different superscript letters within a column indicate significant differences ($p < 0.05$).

4.5. Conclusions

The major findings of this study suggest that biological methods such as germination and fermentation are very effective methods to decrease the immunoreactivity of allergens of mustards seeds. Sandwich ELISA assay demonstrated that five-day germination resulted in 87% reduction in immunoreactivity which means 0.90 log reduction. Similarly, 86% or 0.86 log reduction in immunoreactivity was observed for fermentation at 35ºC. Alteration in mustard protein structure as demonstrated by FTIR results may have led to unmasking, masking or

epitope destruction resulting in changes of immunogenic response as observed in ELISA immunoassay. The conventional cooking of 5% slurry extract of these treated samples at 100ºC demonstrated further reduction in immunoreactivity from 26.61 ppm to 0.480 ppm that is approximately 1.68 log reduction and percentage reduction in immunoreactivity was beyond 98%. This study also proposes that these processing methods are beneficial as these methods resulted in increased antioxidant activities in germinated and fermented mustard seeds. The use of such bio-processing methods should provide good incentives as non-thermal alternatives for allergen reducing and antioxidant enhancing concepts for mustard processing which when further be combined with other procedures such as cooking resulting in further reducing in immunoreactivity of mustard allergens

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CHAPTER V
GENERAL CONCLUSIONS, CONTRIBUTIONS TO KNOWLEDGE AND RECOMMENDATIONS FOR FUTURE WORK

5.1. General Conclusions

In the present study, thermal processing, germination and fermentation were evaluate to understand the immunoreactivity reduction of mustard seeds (*Sinapis alba*) allergens. All investigations demonstrated positive results in reducing immunoreactivity of yellow mustard allergens. Fourier transform infrared (FTIR) spectroscopy demonstrated shifts in wavenumbers (cm^{-1}) and secondary structure changes caused by these processing technologies. Considering effect on quality attributes showed encouraging outcomes along with understanding immunoreactivity reduction of mustard allergen.

With enhanced thermal treatment nearly 4 log reduction (99.97 % reduction) in immunoreactivity was achieved with F_0 value ranging between 3-10 min, whereas conventional cooking up to 60 min did not resulted in reduction beyond 67%. Alteration of protein structure as recorded by FTIR analysis may have resulted in changes of antigenic properties of mustard allergic proteins. These intense thermal treatments resulted in slight changes in mustard slurry extract. Therefore, there is high potential to develop optimized thermal processing parameters which reduce immunoreactivity to ppb concentrations which impairing the product quality, especially for products in liquid or sauce format.

Germination and fermentation were very successful processes to decrease the allergen immunoreactivity of mustard seeds. Five-day germination at 35-40ºC caused 87% reduction in immunoreactivity. Fermentation for 3 days at 35ºC and 25 ºC resulted in 86% and 85% reduction respectively, and when combined together, they demonstrated further reduction in immunoreactivity reaching up to one log reduction which is nearly 90% reduction in immunoreactivity. FTIR analysis confirmed that shifting in wavenumbers in amide I and II region and secondary structure changes due to unmasking, masking or epitope destruction resulting in changes of immunogenic response. The conventional cooking of 5% slurry extract of these treated samples at 100ºC demonstrated even further reduction in immunoreactivity from 26.61 ppm to 0.48 ppm and percentage reduction in immunoreactivity was beyond 98% with log reduction of 1.74. The study also demonstrated increasing antioxidant capacities and total

phenols content resulting in highest amount in samples prepared after treatment by both the processes.

Conclusively, this research is a novel approach to study fate of allergens after applications of selected food processing technologies. It is a clear step forward in understanding different processing techniques for reducing the immunoreactivity of allergens which might turn out to be a boon to the population that are allergic to mustard proteins.

5.2. Contributions to Knowledge

1. This study clearly showed that temperature and time combinations of enhanced thermal processing can be a useful way to optimize thermal processing parameters for allergen immunoreactivity reductions with minor effects on quality attributes.
2. For the first time, germination and fermentation processing methods were demonstrated to have a link with immunoreactivity reduction. The combination of these treatments with domestic cooking can be effective in reducing the immunoreactivity of yellow mustard allergens while increasing antioxidant capacities and total phenol content.

5.3. Recommendations for Future Studies

The current study recommends applying selected food processing methods in various food industry applications such as preparation of sauces, curries, condiments, salad dressings, or flavoring powders to name a few. It is very encouraging study with minimal effect on quality parameters and increased antioxidant capacities and total phenol content. While it is very promising to find significant reduction in immunoreactivity of allergens, it should be recognized that this decrease cannot be assumed to reduce the allergenicity by the same level. This study recommends further confirmation of these results with human trials.